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Genome-wide Search of Oncogenic Pathways Cooperating With ETS Fusions in Prostate Cancer

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14. ABSTRACT Gene fusions involving ETS family transcription factors (TFs) (mainly ERG and ETV1) have been identified in ~50% of prostate cancer cases. To address their roles in prostate cancer, we generated multiple Tmprss2-ETS knockin mouse models. The goal of this project is to identify oncogenic pathways cooperating with ETS fusions using these knockin models. During the second year, we continued our characterization of these knockin models. In addition to our previous observation that Tmprss2-ETS fusions cooperate with Pten-loss, but not with Nkx3.1-loss, to promote development of localized prostate cancer, we found that genes deleted in the interstitial region between Tmprss2 and Erg may cooperate with Pten-loss, or Pten-loss plus ectopic Tmprss2-Erg expression, leading to development of invasive prostate cancer. Furthermore, by focusing on another ETS family TF, ETV6, which is deleted in some prostate cancer cases (including TMPRSS2-ERG fusion positive case), we found that it is also a tumor suppressor in prostate cancer. Overall, the work we have performed so far has identified two additional oncogenic pathways induced by loss of genes in the Tmprss2/Erg interstitial region or by loss of ETV6 that may cooperate with Pten-loss or Tmprss2-Erg fusion plus Pten-loss, in leading to aggressive prostate cancer. Future work will focus on identification of tumor suppressor(s) in the interstitial region, oncogenic program driven by ETV6-loss, and identification of any additional Tmprss2-ETS-cooperating oncogenic pathways.					
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## INTRODUCTION:

During prostate tumorigenesis, multiple oncogenic pathways cooperate to drive disease progression. Identification of these cooperating pathways is the key for defining novel therapeutic targets and for combined therapeutic interventions for treating advanced prostate cancer. Gene fusions involving the ETS family transcription factors, particularly *ERG* and *ETV1*, have been identified in at least 40-50% of human prostate cancer cases [1, 2]. Coding regions of these ETS genes are often rearranged to control regions of several androgen-responsive genes, particularly the *TMPRSS2* gene, leading to aberrant expression of ETS genes. In animal models, aberrant *ERG* or *ETV1* expression in mouse prostates under the control of the *Probasin* (*Pb*) promoter [3-6] leads to either a minor phenotype (*i.e.*, PIN lesions) or almost no observable abnormalities. However, ectopic *ERG* expression can cooperate with *Pten* loss to drive prostate cancer development [7, 8]. Consistent with this, in a tissue reconstitution model, lentiviral overexpression of *ERG* in prostate cells collaborates with activation of the PI3K pathway or the androgen receptor (AR) pathway to induce distinct prostate carcinomas. These observations suggest that although aberrant expression of ETS factors alone in prostates is insufficient for prostate cancer, it sensitizes prostate epithelial cells for cooperation with additional oncogenic mutations to drive full-blown prostate cancer. The main hypothesis of this study is that aberrant *ERG* or *ETV1* expression in prostate cells may cooperate with multiple alternative oncogenic pathways to drive prostate tumorigenesis. Since *TMPRSS2-ERG* fusions (particularly those with the interstitial deletion) are highly prevalent in advanced, castration-resistant (CR) prostate tumors [9, 10], understanding cooperative interactions between ETS fusions and other oncogenic pathways would be the key to develop novel combined therapies for treating this deadly disease. Since mouse models with aberrant ETS expression alone do not develop prostate cancer but serve as a sensitized system, the main purpose of this research is to identify additional oncogenic pathways that may cooperate with ETS gene fusions, leading to prostate cancer. This will be achieved by genetic approaches (*e.g.*, genetic screens; crosses with existing mouse models) under the background of various *Tmprss2-ETS* knockin models we have generated. Novel tumor suppressors may be identified from this study that may cooperate with ETS fusions (upon their loss) to drive prostate cancer development, and pathways affected by their loss will be determined.

## BODY:

The major goal of this research project is to identify oncogenic pathways that cooperate with *TMPRSS2-ETS* gene fusions, leading to prostate cancer formation. During the second year of this grant, we mainly focused on testing several candidate tumor suppressors that may cooperate with *Tmprss2-ETS* fusions (under the background of *Pten*-loss) in mouse models. We also established the renal capsule reconstitution system, which was the system originally proposed to perform the genetic screen.

### **Further characterization of the *Tmprss2-ETS* knockin mouse models**

The majority portion of this work was recently published in *Genes & Development* (Baena et al., *Genes Dev.* 27:683-98) and is provided in the *Appendix*. The major conclusion from this study is that both *Tmprss2-ERG* and *Tmprss2-ETV1* gene fusions can cooperate with loss of a single copy of *Pten*, leading to localized cancer [Prostatic intraepithelial neoplasia (PIN) lesions], but only *ETV1* appeared to support development of invasive adenocarcinoma under the background of full *Pten* loss. Furthermore, these two types of ETS fusions appear to cooperate with *Pten*-loss in different ways: *ERG* negatively regulates the androgen receptor (AR) transcriptional program and thus it may cooperate with *Pten*-loss, which also downregulates the AR program, in leading to an immature phenotype and increased proliferation; In contrast, *ETV1* appears to enhance AR signaling and reprogram the metabolism of prostate cells, and it may cooperate with *Pten*-loss by further enhancing metabolic reprogramming, in particular, by favoring steroid biosynthesis, a pathway critical for invasive adenocarcinoma cells. Thus, our initial work has provided mechanistic insights into how different ETS gene fusions cooperate with other oncogenic pathway in leading to development of prostate cancer.

## Test potential contribution of genes deleted in the interstitial region between *Tmprss2* and *Erg* to prostate cancer with *Tmprss2-Erg* gene fusion

The majority of prostate cancer cases with gene rearrangements carry the *TMPRSS2-ERG* fusion [1]. Both *ERG* and *TMPRSS2* are located ~3Mb apart on human chromosome 21. The predominant mechanism to generate the *TMPRSS2-ERG* fusion is the intrachromosomal deletion between these two genes [11-16]. The 3Mb interstitial deletion may lead to haploinsufficiency of one or more genes within this region. In fact, previous studies have suggested that several genes in this region may have tumor/metastasis suppression roles. For example, knockout of one of the genes within this region, *HMGNI*, leads to increased N-cadherin expression [17], which is associated with high-grade prostate cancer [18], and altered G2/M checkpoint [19]. In addition, an ETS family transcription factor gene located in this region, *ETS2*, appears to serve as a tumor suppressor when expressed at an elevated level, as it represses *Apc<sup>min</sup>*-mediated tumors in mouse models of Down Syndrome, but not in normal mice (i.e., three copies of *Ets2* in Down Syndrome mouse models versus two copies of *Ets2* in normal mice) [20]; Both point mutation (e.g., R437C) and focal deletions of *ETS2* have been found in prostate cancer, and overexpression of wild-type *ETS2* (but not R437C-mutant *ETS2*) resulted in decreased migration, invasion and proliferation in VCaP cells [21]. Moreover, an interferon-inducible GTPase, MxA, encoded by *MXI*, another gene located in this interstitial region, was shown to express in the human prostate carcinoma cell line PC-3, but not in its highly metastatic derivative PC-3M [22]; this protein suppresses metastasis by inhibiting tumor cell motility and invasion. Combining these together, it seems that reduced expression of one or more genes within this interstitial region may promote prostate tumor progression and metastasis. To date, clinical data regarding potential involvement of the interstitial deletion in prostate tumorigenesis remains contradictory. In a study involving primary prostate cancers and hormone naïve lymph node metastasis, *TMPRSS2-ERG* rearranged tumors through interstitial deletion (the so-called Edel subtype) were found to significantly associate with higher tumor stage and the presence of metastatic disease involving pelvic lymph nodes [15]. In another study, patients with prostate tumors with a duplication of *TMPRSS2-ERG* in combination with deletion of 5'-*ERG* (the 2+Edel subtype) exhibited poor cause specific survival [23]. Furthermore, it has been reported that in androgen-independent metastatic lethal prostate cancers, all metastatic sites harboring the *TMPRSS2-ERG* fusion were associated with the Edel subtype [24]. These studies strongly suggest that the Edel subtype of *TMPRSS2-ERG* fusion is a distinct and aggressive molecular subtype of prostate cancer.

In our two *Tmprss2-Erg* knockin models, one was generated by knocking in the N-terminus-truncated *Erg* cDNA directly to the mouse *Tmprss2* locus (thus without the interstitial deletion, referred to as the *T-ERG* model), the other was generated by Cre-mediated excision of the interstitial region and subsequent rearrangement of the *Erg* coding region to the *Tmprss2* promoter (*T-3Mb-Erg* model before excision, *T-Δ-Erg* after excision of the interstitial region). Under the total *Pten*-loss background (in *Pb-Cre;Pten<sup>LL</sup>* mice), we found that these two knockin models gave rise to very different phenotypes: *Pb-Cre;T-3Mb-Erg;Pten<sup>LL</sup>* males developed large poorly-differentiated prostate tumors and locally invasive prostate adenocarcinomas (invasive ducts) by 12-month of age (n=6), whereas *Pb-Cre;T-ERG;Pten<sup>LL</sup>* males only developed high-grade PIN lesions (HGPIN) (n=4) that were not invasive and were well-differentiated at 12-month of age, similar to those of *Pb-Cre;Pten<sup>LL</sup>* control males (but with even less stromal cells, Figure 1A). Interestingly, when we checked ectopic *Erg* expression in these lesions by immunohistochemical staining, we found that while *Erg* was robustly expressed in HGPIN lesions from the *Pb-Cre;T-ERG;Pten<sup>LL</sup>* males, in *Pb-Cre;T-3Mb-Erg;Pten<sup>LL</sup>* males, *Erg* was expressed in a mosaic pattern in invasive ducts and was often not expressed in poorly differentiated prostate tumors (Figure 1A). To determine whether the prostate lesions in this model have undergone *Pb-Cre*-mediated excision of the 3Mb interstitial region and generation of the *Tmprss2-Erg* gene fusion, we performed laser-capture microdissection to collect cancerous tissues from various regions of the *Pb-Cre;T-3Mb-Erg;Pten<sup>LL</sup>* prostates and performed PCR on their genomic DNA; we found that the *Tmprss2-Erg* gene fusion with the interstitial deletion was indeed generated successfully (Figure 1B). The reason that *Erg* expression was not detected in many prostate lesions in this model could be due to regulation of *Erg* expression by the endogenous *Tmprss2* promoter, which is subject to negative regulation by *Erg*. Overall, our data thus provides a strong genetic evidence to support that the interstitial deletion indeed contributes to development of aggressive

prostate cancer under the *Pten*-null background, and that one or more genes in this region may be tumor suppressor(s) in prostate cancer.

### **Test whether *Tmprss2-ETS* fusions cooperate with *Nkx3.1*-loss**

Previously we described our initial test to determine whether *Tmprss2-ETS* fusions cooperate with *Nkx3.1*-loss. The initial conclusion was that *Nkx3.1*<sup>+/-</sup> alone does not appear to cooperate with ETS fusions. In a larger cohort of male mice, including *Nkx3.1*<sup>+/-</sup> (n=9), *Tmprss2 $\Delta$ Erg*; *Nkx3.1*<sup>+/-</sup> (*T- $\Delta$ Erg*; *Nkx3.1*<sup>+/-</sup>, i.e., *Tmprss2-Erg* fusion with interstitial deletion, n=9), *Tmprss2-ERG*; *Nkx3.1*<sup>+/-</sup> (*T-ERG*; *Nkx3.1*<sup>+/-</sup>, n=10), and *Tmprss2-ETV1*; *Nkx3.1*<sup>+/-</sup> (*T-ETV1*; *Nkx3.1*<sup>+/-</sup>, n=5), we further confirmed that there seems no evidence to support any cooperation between *Tmprss2-ETS* fusions and *Nkx3.1*<sup>+/-</sup> (Figure 2A); among all these males, only one developed HGPIN lesions, which was an 18-month old *T- $\Delta$ Erg*; *Nkx3.1*<sup>+/-</sup> mouse.

Under the *Pten*<sup>+/-</sup>; *Nkx3.1*<sup>+/-</sup> background, we did observe cooperation of *Tmprss2-ETS* fusions with this double mutant background for the development of PIN lesions (Figure 2B). However, the frequency of developing PIN lesions was not significantly increased over those under the *Pten*<sup>+/-</sup>-only background (Figure 2C), thus suggesting that the observed cooperation between *Tmprss2-ETS* fusions and *Pten*<sup>+/-</sup>; *Nkx3.1*<sup>+/-</sup> was mainly due to *Pten*-loss rather than due to *Nkx3.1*-loss alone.

### **Test ETV6 as a potential tumor suppressor in prostate cancer**

ETV6 (also known as TEL) is another ETS family transcription factor and it undergoes hemizygous deletion in about 25% of prostate cancers, suggesting it may function as a tumor suppressor in prostate cancer [25]. Furthermore, a partial deletion of *ETV6* was observed in a hormone naïve metastatic lymph node sample from a *TMPRSS2-ERG* fusion-positive prostate cancer patient [25]. This gene was therefore included in our original proposal as a candidate tumor suppressor to test in our genetic screen. We investigated a potential tumor suppressor role of this ETS factor in prostate tumorigenesis by breeding a conditional knockout allele of *Etv6* (*Etv6*<sup>L</sup>) (from Dr. Stuart Orkin's group [26]) to the *Pb-Cre*; *Pten*<sup>L/L</sup> background. We found that although *Etv6*-loss alone did not appear to affect prostate cells significantly, it cooperated with full *Pten*-loss (e.g., in *Pb-Cre*; *Etv6*<sup>L/+</sup>; *Pten*<sup>L/L</sup> males), leading to development of locally invasive prostate adenocarcinoma [e.g., loss in smooth muscle actin (SMA) staining and presence of invasive ducts (Figure 3)] by 12-months of age (n=6); Biallelic *Etv6* loss did not appear to be more aggressive than single copy loss so far (n=3). This data thus suggests that ETV6 is indeed a tumor suppressor in prostate cancer, and its hemizygous deletion is already sufficient to contribute to prostate tumorigenesis, when cooperated with other oncogenic events (e.g., *Pten*-loss). As *TMPRSS2-ERG* drives an oncogenic program that partially overlaps with that of *Pten*-loss, we suspect *ETV6*-loss may also cooperate with this fusion, either alone or together with *Pten*-loss.

### **Renal capsule reconstitution assay**

After obtained our animal protocol approval, and after training and certifying (by our animal facility) personnel in our lab to perform castration and renal capsule transplantation surgical procedures, we have established the renal capsule reconstitution assay and performed the initial experiment by transplanting wild type (WT), or *T-ETV1*, or *T-ERG* prostate cells (lineage-negative cells enriched by MACS beads) infected with shRNA lentivirus for *Pten* to recipient mice (Figure 4). Compared to WT, we saw certain degree of cooperation between *T-ETV1* and *Pten* knockdown, and only very slight cooperation (if any) between *T-ERG* and *Pten* knockdown. This could be due to the fact that ETV1 is robustly expressed from the *T-ETV1* knockin allele whereas ERG is only weakly expressed from the endogenous *Tmprss2* promoter from the *T-ERG* knockin allele, as described in our previous progress report and in the attached paper here. Currently we are optimizing this assay. One concern is that due to the weaker phenotype and potentially insufficient material for further analysis, we may need to choose an alternative approach for genetic screen.

### **Future plans**

1. As stated in the previous progress report, recent progress in the field suggested that the renal capsule reconstitution system may not be the most ideal system for searching for ETS-cooperating pathways, as

this assay is heavily biased toward basal cells (i.e., prostate luminal cells cannot engraft well in this assay), and although both luminal and basal cells could serve as cells of origin of prostate cancer, luminal cells could initiate prostate cancer much more effectively than basal cells, and that the barrier for basal cells to serve as the cellular origin was the need for them to differentiate into luminal cells first [27]. Thus, it appears that prostate luminal cells may serve as the major cellular origin for prostate cancer. We therefore more favor a strategy to search for ETS-cooperating mutations in prostate luminal cells. Furthermore, characterization of our *Tmprss2-ETS* knockin models so far suggested that *Tmprss2-ETS* fusions, in particular, *Tmprss2-Erg* fusions, are only weakly oncogenic, making it difficult to perform the genetic screen using our originally proposed renal capsule reconstitution approach. As stated in the previous report, we now more favor the alternative approach for the genetic screen by using the potentially more sensitive insertional mutagenesis approach based on the *Sleeping Beauty* (*SB*) transposon-based somatic mutagenesis system. Currently we are in the process of signing the MTAs with NCI MMHCC repository and will import the *SB* mouse lines to our colony for breeding with *Pb-Cre* (to activate mobilization of the *SB* transposon in prostate cells) and our *Tmprss2-ETS* conditional knockin models.

2. We are further optimizing our renal capsule reconstitution system to determine whether it is suitable for the initially proposed genetic screen, at least for the *T-ETV1* knockin prostate cells.
3. We will wrap up the (negative) data for *Nkx3.1* cooperation with *Tmprss2-ETS* fusions (including mice under the *Nkx3.1*<sup>-/-</sup> background) and publish this work.
4. We will test whether loss of *Etv6* cooperates with *Tmprss2-ETS* fusions, either alone or under the *Pten*-loss background; we will also determine at the molecular level how loss of *Etv6* contributes to prostate tumorigenesis.
5. We will determine any potential tumor suppressor(s) in the interstitial region between *Tmprss2* and *Erg* that may contribute to the cooperation between the interstitial deletion and either the *Pten*-loss alone or *Pten*-loss plus the *Tmprss2-Erg* fusion.

## KEY RESEARCH ACCOMPLISHMENTS:

1. Finished our characterization of all three *Tmprss2-ETS* knockin mouse models, and published our initial work in *Genes and Development*.
2. Obtained the initial genetic evidence to support that the interstitial deletion in *TMPRSS2-ERG* fusion positive cases may actually contribute to development of advanced prostate cancer.
3. Obtained the genetic evidence to support that ETV6 is a tumor suppressor in prostate cancer.
4. Completed the study to show that loss of *Nkx3.1* is not sufficient to cooperate with all *Tmprss2-ETS* fusions for the development of prostate cancer.
5. Established the renal capsule reconstitution system for prostate cells.

## REPORTABLE OUTCOMES:

### Manuscripts:

1. Baena E, Shao Z, Linn DE, Glass K, Hamblen MJ, Fujiwara Y, Kim J, Nguyen M, Zhang X, Godinho FJ, Bronson RT, Mucci LA, Loda M, Yuan GC, Orkin SH, Li Z. ETV1 directs androgen metabolism and confers aggressive prostate cancer in targeted mice and patients. *Genes Dev.* 2013;27(6):683-98. PMID: PMC3613614.

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## CONCLUSION:

In conclusion, we have thoroughly characterized three *Tmprss2-ETS* knockin mouse models we generated. We found that although ectopic expression of ERG or ETV1 sensitizes prostate cells for cooperation with oncogenic pathways, such as activation of the PI3-K pathway (due to *Pten* loss), they do not appear to cooperate with the oncogenic pathway due to *Nkx3.1* loss. In addition to these observations, we found that genes deleted in the interstitial region between *Tmprss2* and *Erg* appear to cooperate with *Pten*-loss, or both *Pten*-loss and ectopic *Tmprss2-Erg* expression, leading to development of invasive prostate cancer. Furthermore, by focusing on another ETS family transcription factor, ETV6, which is deleted in some prostate cancer cases (including *TMPRSS2-ERG* fusion positive case), we found that ETV6 is a tumor suppressor in prostate cancer; although loss of *Etv6* alone did not seem to affect prostate cells, loss of one copy of *Etv6* was sufficient to cooperate with *Pten*-loss, leading to development of invasive prostate cancer. In summary, the work we have performed so far has identified two additional oncogenic pathways induced by loss of gene(s) in the *Tmprss2/Erg* interstitial region or by loss of *Etv6* that contribute to aggressive prostate cancer. Future work will focus on identification of tumor suppressor(s) in the interstitial region, understanding the oncogenic program induced by the *Etv6*-loss, as well as identification of any additional *Tmprss2-ETS*-cooperating oncogenic pathways by a genetic screen based on the *SB* transposon insertional mutagenesis system. The knowledge from this study (e.g., whether any tumor suppressor in the interstitial region contributes to fusion-positive prostate cancer) will provide novel insights into prevention, diagnosis and treatment of prostate cancer.

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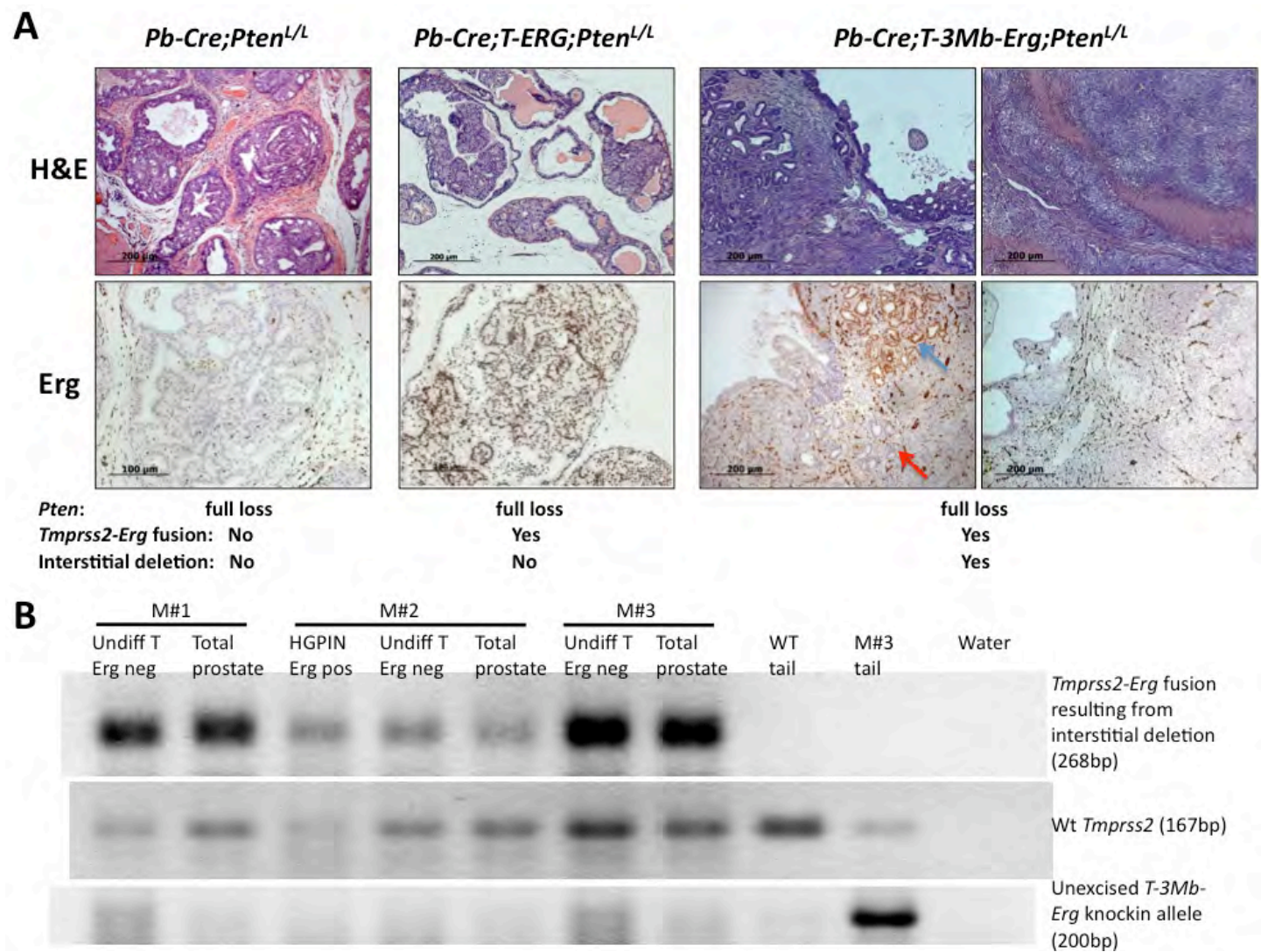
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## APPENDICES:

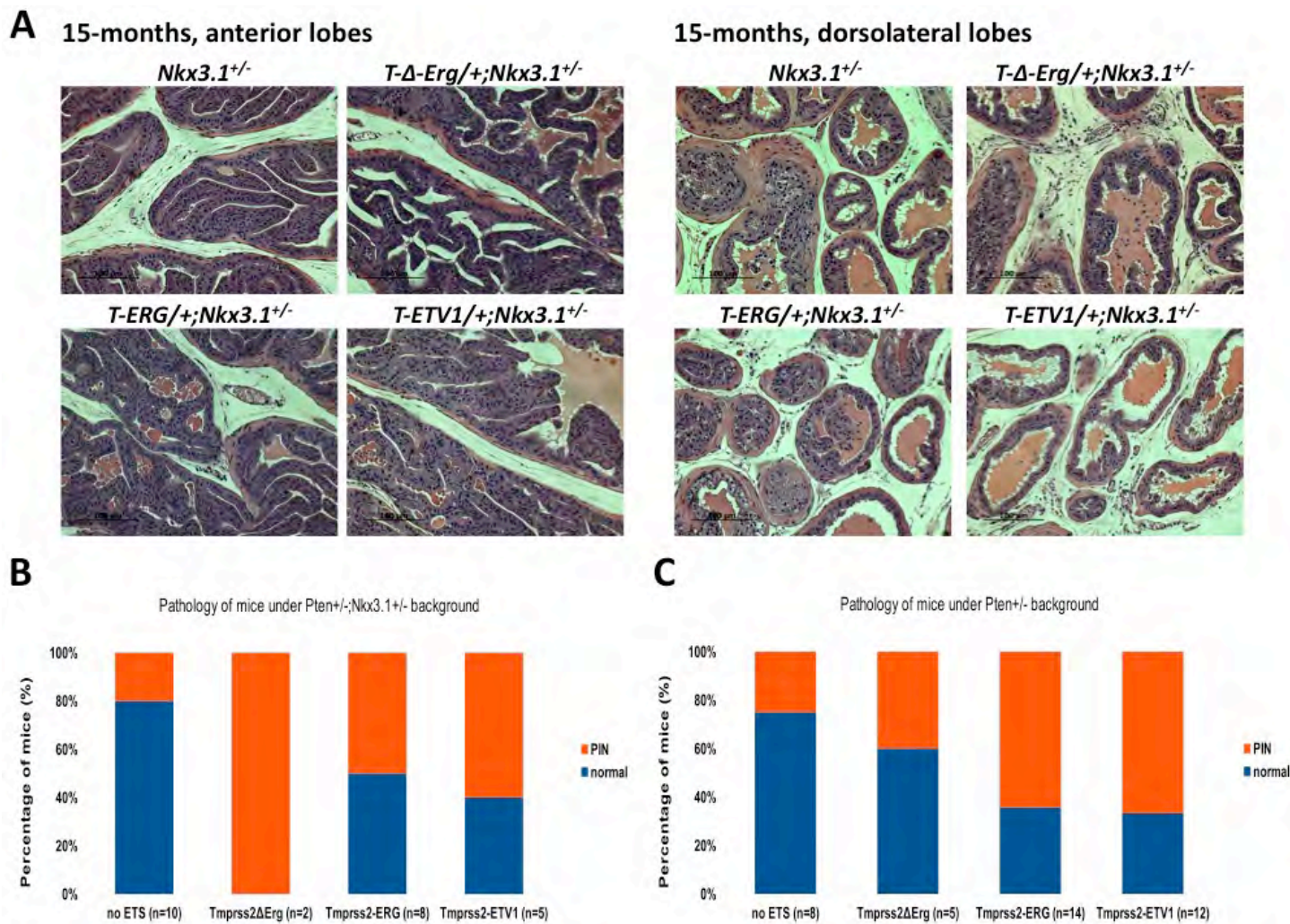
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SUPPORTING DATA:

Figures 1-4

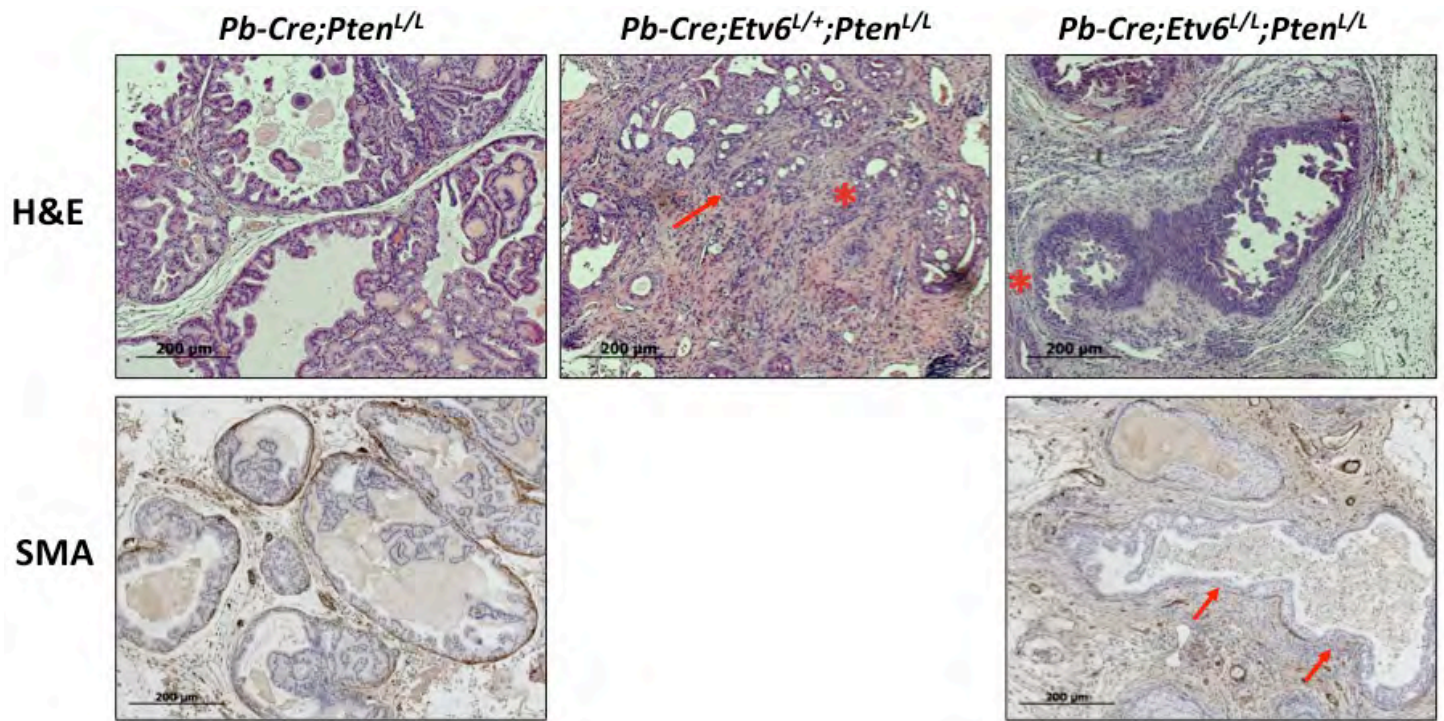


**Figure 1. Loss of gene(s) in the interstitial region between *Tmprss2* and *Erg* may contribute to advanced prostate cancer.** (A) Top row shows hematoxylin & eosin (H&E) staining of prostate sections from male mice with the indicated genotypes. The two pictures for *Pb-Cre;T-3Mb-Erg;Pten<sup>L/L</sup>* males show invasive ducts (left) and undifferentiated tumors (right) in their prostates. Lower row shows immunohistochemical staining of Erg protein. In *Pb-Cre;Pten<sup>L/L</sup>* control section, Erg staining was only found in endothelial and hematopoietic cells; In *Pb-Cre;T-ERG;Pten<sup>L/L</sup>* section, Erg staining was found in many prostate cells in the HGPIN lesion; In *Pb-Cre;T-3Mb-Erg;Pten<sup>L/L</sup>* sections, Erg staining was found in a portion of prostate cells in the invasive ducts (blue arrow, left picture), but not in other similarly looking ducts (red arrow, left picture), and Erg staining was also not found in prostate epithelial cancer cells in the undifferentiated tumors (right picture, Erg+ cells here were endothelial and hematopoietic cells). (B) PCR analysis of genomic DNA isolated from different regions of the *Pb-Cre;T-3Mb-Erg;Pten<sup>L/L</sup>* prostate tumors by laser-capture microdissection confirming creation of the *Tmprss2-Erg* fusion by Cre-mediated deletion of the interstitial region. The un-rearranged WT copy of *Tmprss2* was used as the internal control. M#1, M#2, and M#3 were three individual *Pb-Cre;T-3Mb-Erg;Pten<sup>L/L</sup>* males that developed prostate tumors; Undiff T: undifferentiated tumor; Erg neg: negative for Erg staining; Erg pos: positive for Erg staining.

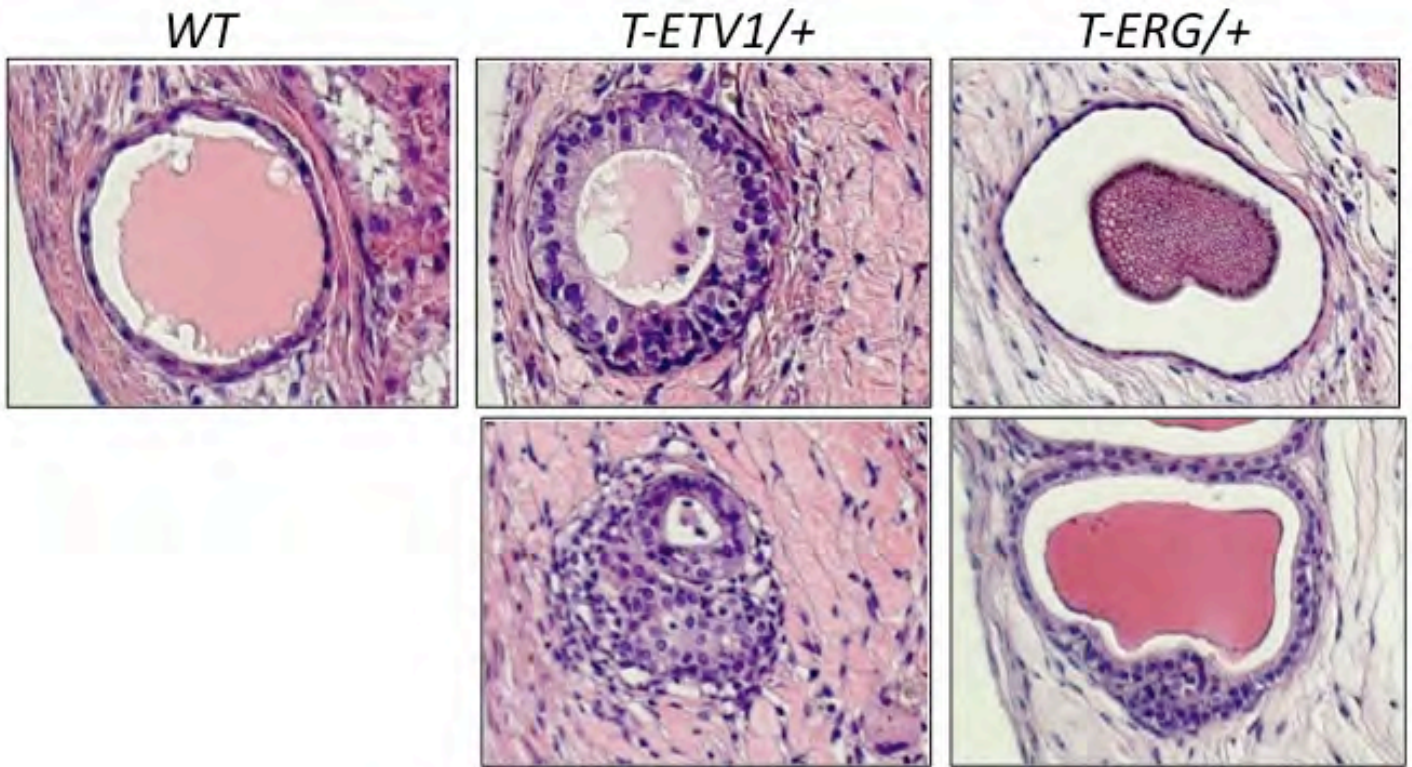


**Figure 2. Loss of one copy of *Nkx3.1* does not cooperate with *Tmprss2-ETS* fusions.** (A) Representative normal-looking anterior prostate lobes (left) and dorsolateral prostate lobes (right) from 15-month old males of the indicated genotypes. (B) All three *Tmprss2-ETS* knockin alleles cooperate with *Pten*<sup>+/-</sup>; *Nkx3.1*<sup>+/-</sup>; however, they also cooperate with *Pten*<sup>+/-</sup> alone in a similar manner (C).





**Figure 3. *Etv6*-loss cooperates with *Pten*-loss to drive development of invasive prostate cancer.** Top row shows H&E staining of prostate sections from male mice with the indicated genotypes. Arrow: invasive ducts; Stars: locally invasive prostate cancers. Lower row shows immunohistochemical staining of smooth muscle actin (SMA), which is a basal marker. Arrows: regions with loss of SMA+ basal cells (a sign of invasive prostate cancer).



**Figure 4. Renal capsule reconstitution of transplanted prostate cells.** Prostate cells with the indicated genotypes were infected by lentivirus expressing shRNA for *Pten* before transplantation. *Pten* knockdown appears to cooperate with *T-ETV1* knockin (middle).



## ETV1 directs androgen metabolism and confers aggressive prostate cancer in targeted mice and patients

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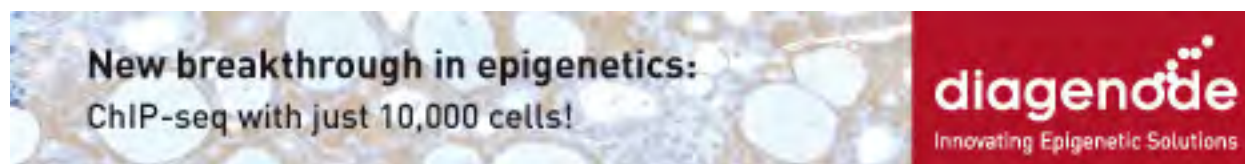
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# ETV1 directs androgen metabolism and confers aggressive prostate cancer in targeted mice and patients

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**Distinguishing aggressive from indolent disease and developing effective therapy for advanced disease are the major challenges in prostate cancer research. Chromosomal rearrangements involving ETS transcription factors, such as ERG and ETV1, occur frequently in prostate cancer. How they contribute to tumorigenesis and whether they play similar or distinct in vivo roles remain elusive. Here we show that in mice with ERG or ETV1 targeted to the endogenous *Tmprss2* locus, either factor cooperated with loss of a single copy of *Pten*, leading to localized cancer, but only ETV1 appeared to support development of invasive adenocarcinoma under the background of full *Pten* loss. Mechanistic studies demonstrated that ERG and ETV1 control a common transcriptional network but largely in an opposing fashion. In particular, while ERG negatively regulates the androgen receptor (AR) transcriptional program, ETV1 cooperates with AR signaling by favoring activation of the AR transcriptional program. Furthermore, we found that ETV1 expression, but not that of ERG, promotes autonomous testosterone production. Last, we confirmed the association of an ETV1 expression signature with aggressive disease and poorer outcome in patient data. The distinct biology of ETV1-associated prostate cancer suggests that this disease class may require new therapies directed to underlying programs controlled by ETV1.**

[**Keywords:** ERG; ETS transcription factor; ETV1; *Pten*; androgen receptor; metabolism]

Supplemental material is available for this article.

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Prostate cancer is a heterogeneous disease. Recent studies show little benefit from prostate-specific antigen (PSA) screening and radical prostatectomy for men with lower-risk disease (Wilt et al. 2012). A central challenge in management is identification of those men with prostate cancer whose disease will eventually progress to the lethal castration-resistant stage. Understanding molecular events leading to castration-resistant prostate cancer (CRPC) is

critical for the development of improved therapies for such patients.

Chromosomal rearrangements involving genes encoding ETS transcription factors (notably, ERG and ETV1) are found in ~50% of human prostate cancer cases and likely constitute the most frequent gene rearrangements in human malignancies (Tomlins et al. 2005; Gopalan et al. 2009). The translocations place the coding regions of *ERG* or *ETV1* under the control of androgen-responsive promoters, such as *TMPRSS2*, thereby activating expression in response to androgens. *TMPRSS2* has been reported as the principal 5' fusion partner of *ERG*, whereas more heterogeneous 5' fusion partners, such as *TMPRSS2*, *SCL45A3*, or *ACSL3*, have been described for *ETV1* (Tomlins et al. 2007; Attard et al. 2008b). The majority of these 5' fusion

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partners are also androgen-responsive genes. As ETS proteins, ERG and ETV1 are involved in regulation of cell growth, proliferation, differentiation, and apoptosis through activation or repression of target genes (Oikawa and Yamada 2003). Although functional overlap among different members of the ETS family exists, individual ETS factors also serve distinct roles. Thus, the expression pattern of ETS members through development varies, along with their repertoire of target genes, biological processes regulated, and oncogenic potentials (Seth and Watson 2005; Kunderfranco et al. 2010; Wei et al. 2010; Hollenhorst et al. 2011).

Clinical studies of the prevalence and prognostic significance of ETS fusions in prostate cancer have yielded discrepant results, possibly related to differences in the genetics of the evaluated populations and diversity in methods used. Several studies suggest that ETS fusions are associated with a worse prognosis (Demichelis et al. 2007; Nam et al. 2007; Attard et al. 2008a), whereas others have failed to confirm the correlation (Gopalan et al. 2009; Hermans et al. 2009; Minner et al. 2011). Cases with ETS fusions are generally grouped together for patient stratification. However, considering all ETS translocations as a single entity risks obscuring possible differences in the contribution of each to disease outcome. For example, effects of *TMPRSS2-ERG*, the most common translocation, may bias findings of aggregate studies. Studies to date have not specifically addressed the biology of individual ETS fusions and their associated outcomes.

In this study, we used knock-in mouse modeling and comprehensive genome-wide approaches to characterize the functional specificities of ERG and ETV1 in prostate cancer. Our data indicate that ERG and ETV1 regulate a common set of genes, such as androgen receptor (AR) target genes, but in an opposing direction. In particular, ETV1, but not ERG, up-regulates expression of AR target genes as well as genes involved in steroid biosynthesis and metabolism. This ETV1-driven oncogenic program predisposes prostate cells for cooperation with other oncogenic events, such as PTEN loss, leading to more aggressive disease in murine models and human patients. Our findings further establish different biological subtypes of human prostate cancer based on distinct ETS factor-driven signatures.

## Results

### *Tmprss2-ETV1 cooperates with total Pten loss, leading to invasive adenocarcinoma*

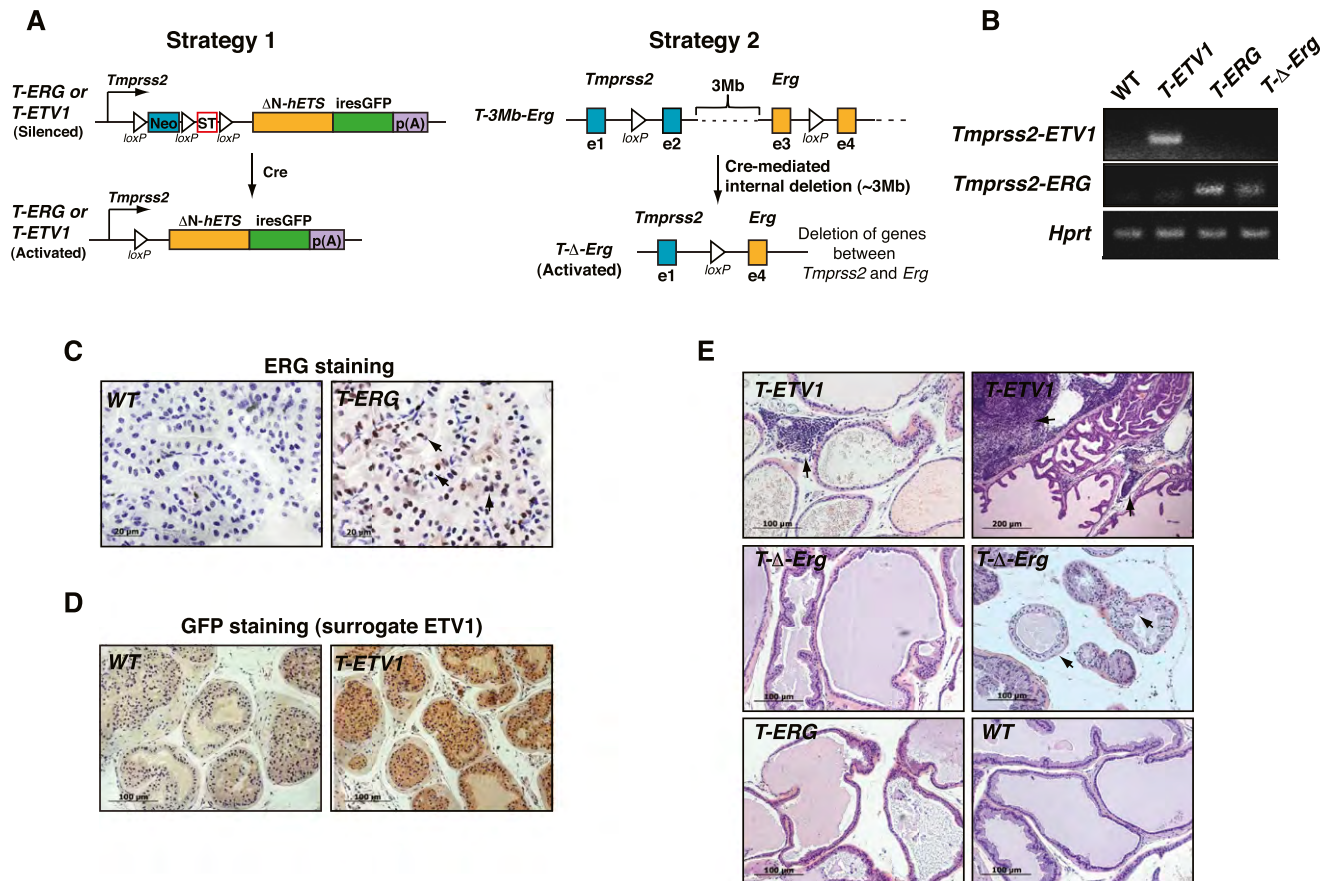
As a step toward defining the roles of ETS fusions in prostate cancer, several groups have generated transgenic mouse strains that express ERG or ETV1 ectopically under the control of the *Probasin* (*Pb*) promoter (*Pb-ERG* or *Pb-ETV1*) (Tomlins et al. 2007, 2008; Klezovitch et al. 2008; Carver et al. 2009; King et al. 2009; Shin et al. 2009). Interpretation of results has varied. Prostatic intraepithelial neoplasia (PIN)-like lesions have been described in prostates of *Pb-ERG* and *Pb-ETV1* transgenic males

(Tomlins et al. 2007, 2008; Klezovitch et al. 2008; Shin et al. 2009). However, others have reported that *Pb-ERG* transgenic males are normal (Carver et al. 2009; King et al. 2009). Discrepant findings may be related to mouse strain differences, to different transgene integration sites, or in the precise portions of the ETS cDNAs that were expressed. We reasoned that mice engineered to express ETS factors from an endogenous promoter in the proper chromosomal configuration might provide a more relevant biological context. Moreover, prior transgenic models cannot address potential contributions of haploinsufficiency or loss of genes deleted between *TMPRSS2* and *ERG* to prostate tumorigenesis, such as occurs in patients with a *TMPRSS2-ERG* fusion generated through an interstitial deletion of chromosome 21.

We engineered knock-in mouse models to recapitulate *TMPRSS2-ETS* fusions (with or without the interstitial deletion) in prostate cancer. We used two strategies. In the first strategy, we knocked in N terminus-truncated human *ERG* or *ETV1* cDNA, together with an *ires-GFP* cassette, into exon 2 of the mouse *Tmprss2* locus (referred to as *T-ERG* or *T-ETV1* hereafter), which shares ~80% homology as well as at least two conserved AR-binding sites with those of the human *TMPRSS2* (Fig. 1A; Supplemental Fig. S1; Jacquinet et al. 2000). The resultant fusion transcripts recapitulate the *TMPRSS2-ERG*<sub>a</sub> or *TMPRSS2-ETV1*<sub>a</sub> fusions in patients (Tomlins et al. 2005). In the second strategy, we used sequential gene targeting to introduce *loxP* sites into the *Tmprss2* and *Erg* loci on the same chromosome (Fig. 1A; Supplemental Fig. S2A,B). Cre-mediated recombination deletes the ~3-Mb intragenic region and generates the *Tmprss2-Erg* fusion gene (Supplemental Fig. S2C,D), which approximates the *TMPRSS2-ERG*<sub>a</sub> fusion subtype (Tomlins et al. 2005). Since most genes in this interstitial region are syntenic between humans and mice (Supplemental Fig. S2E), this unique knock-in model also permits assessment of the contribution of the interstitial deletion to prostate cancer development (referred to as *T-3Mb-Erg* or *T-Δ-Erg* before or after Cre-mediated excision of the interstitial region, respectively) (Fig. 1A). In all three knock-in alleles (i.e., *T-ETV1*, *T-ERG*, and *T-Δ-Erg*) we confirmed expression of their corresponding fusion transcripts in prostates (Fig. 1B). By immunohistochemistry (IHC), we detected moderate expression of Erg protein in the knock-in prostates (Fig. 1C). Despite multiple efforts, we were unable to validate an antibody that faithfully detects ETV1 protein by IHC. Therefore, we used IHC staining for GFP as a surrogate for ETV1 expression, as the knock-in GFP reporter is under the same transcriptional control (Fig. 1A). With this approach, we detected robust GFP (ETV1) expression in prostate epithelial cells but not in stromal cells (Fig. 1D). In all three knock-in strains, prostates appeared largely normal, and we did not observe prostatic intraepithelial neoplasia (PIN) lesions or cancer (Fig. 1E). However, in a portion of *T-ETV1* males (four out of 11), in particular those at old ages (≥18 mo; three out of three), we observed varying degrees of inflammation (Fig. 1E). In addition, pathological analysis in several exceptional *T-Δ-Erg* males (four out of 21 but in none of the *T-ERG*



## ETV1 promotes aggressive prostate cancer phenotype

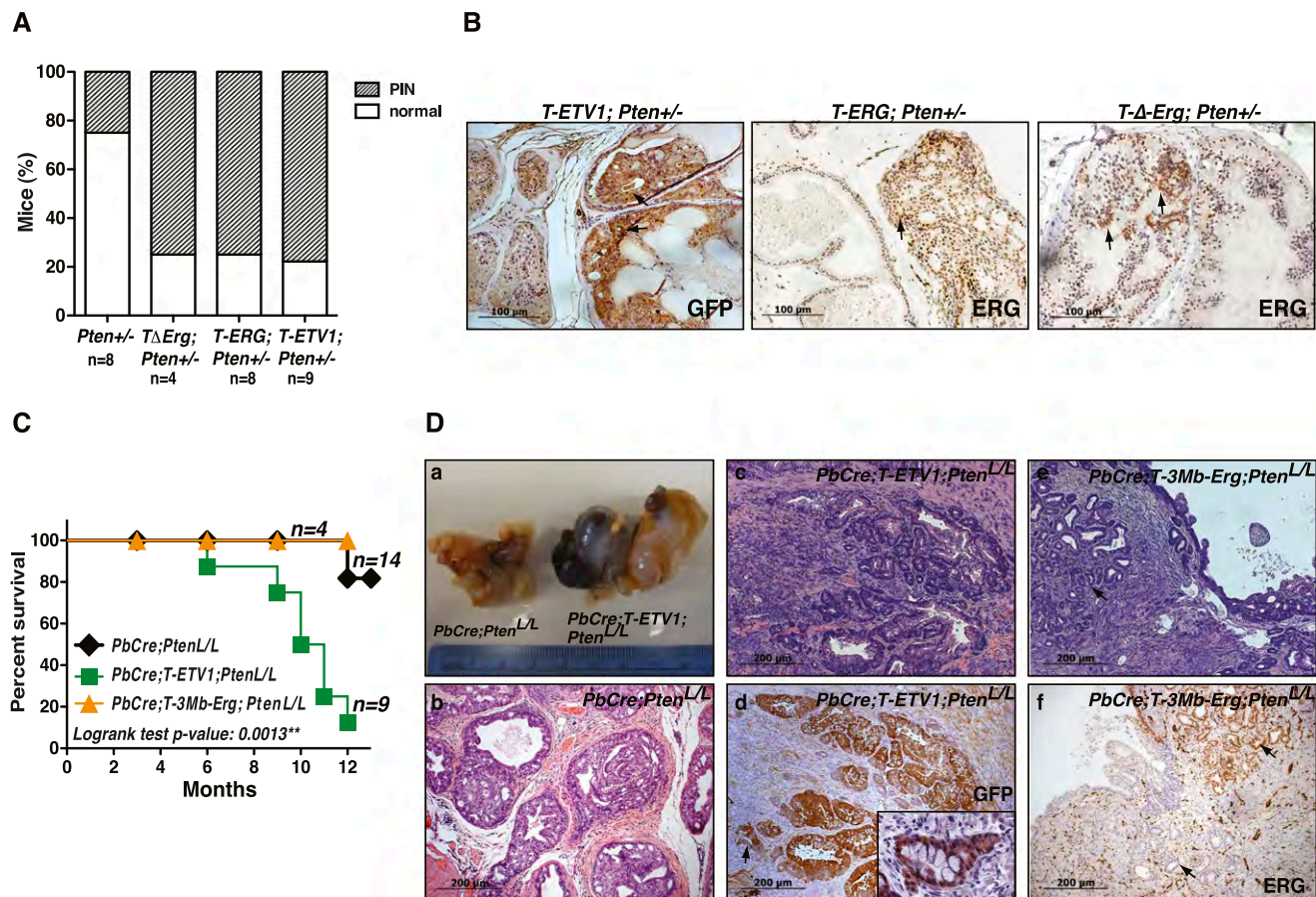


**Figure 1.** *Tmprss2-ERG* (with or without interstitial deletion) and *Tmprss2-ETV1* expression are insufficient to initiate prostate tumorigenesis. (A) Targeting strategies for engineering *Tmprss2-ERG* and *Tmprss2-ETV1* knock-ins. Strategy 1 is based on direct knock-in of N terminus-truncated human *ERG* or *ETV1* cDNA ( $\Delta N$ -hETS) into the murine *Tmprss2* locus. Strategy 2 is based on the introduction of *loxP* sites to murine *Tmprss2* and *Erg* loci by sequential gene targeting in mouse embryonic stem cells so that the 3-Mb interstitial region can be deleted by Cre-mediated recombination and meanwhile generate the *Tmprss2-Erg* gene fusion. Details of gene targeting are shown in Supplemental Figures S1 and S2. (B) RT-PCR showing expression of the *Tmprss2-ETV1* fusion transcripts in T-ETV1 knock-in prostates and expression of the *Tmprss2-ERG* fusion transcripts in T-ERG and T-Δ-*Erg* knock-in prostates but not in wild-type (WT) prostates. (C) IHC staining showing moderate ERG expression (arrows) in the anterior lobes of a T-ERG knock-in male but not in the wild-type male. (D) IHC staining showing homogeneous GFP expression (as surrogate for ETV1) in the anterior lobes of a T-ETV1 knock-in male but not in the wild-type male. (E) Hematoxylin and eosin (H&E) staining showing normal prostate histology from all three knock-ins (showing ventral lobes except those of T-ETV1). Arrows in T-ETV1 pictures indicate inflammation in T-ETV1 knock-in males ([left] slight inflammation in the lateral lobe of a young knock-in male; [right] extensive inflammation in the anterior lobe of a 30-mo-old knock-in male). (Right) Arrows in the T-Δ-*Erg* picture indicate abnormal-looking (lightly stained "foamy"-looking cytoplasm, randomly distributed nuclei) prostate cells, observed in four out of 21 of T-Δ-*Erg* males. Bars, 100 μm (200 μm in top right picture). All animals analyzed in C–E were ~10 mo of age unless otherwise indicated.

males) revealed some hyperplasia and foci of cells with lightly stained cytoplasm and loss of polarity (Fig. 1E). Despite these minor phenotypes, we conclude that expression of ERG or ETV1 from the endogenous *Tmprss2* promoter, even in the presence of the interstitial deletion (for *Erg* fusion), is insufficient to initiate prostate tumorigenesis.

Overexpression of ERG or ETV1 from the *Pb* promoter or through lentiviral transduction in prostate cells has been previously reported to cooperate with activation of the PI3K pathway to drive a more aggressive prostate cancer phenotype (Carver et al. 2009; King et al. 2009; Zong et al. 2009). To determine whether this is also the case

when ETV1 or ERG is expressed from the endogenous *Tmprss2* promoter, we bred mice containing the knock-in alleles with *Pten*<sup>+/-</sup> mice. We found that within the time frame monitored (up to 15 mo of age), prostates of all aged T-ETV1;*Pten*<sup>+/-</sup>, T-ERG;*Pten*<sup>+/-</sup>, and T-Δ-*Erg*;*Pten*<sup>+/-</sup> double-mutant males developed PIN lesions that stain positive for phosphorylated AKT (pAKT), whereas prostates of *Pten*<sup>+/-</sup>-alone mice appeared largely normal (Fig. 2A; Supplemental Fig. S3). In the above cohort, PIN lesions from double-mutant males maintained relatively uniform and high levels of ETV1 (GFP) or ERG expression (Fig. 2B); this is particularly notable for ERG, as in the prostates of ERG knock-in alone, ERG expression was



**Figure 2.** Cooperation of *Tmprss2-ERG* and *Tmprss2-ETV1* gene fusions with *Pten* loss. (A) Bar graph summarizing histology of prostates from *Pten*<sup>+/-</sup>, *T-ΔErg*; *Pten*<sup>+/-</sup>, *T-ERG*; *Pten*<sup>+/-</sup>, and *T-ETV1*; *Pten*<sup>+/-</sup> males. All males were at 6–15 mo of age when checked. The youngest *Pten*<sup>+/-</sup>, *T-ΔErg*; *Pten*<sup>+/-</sup>, *T-ERG*; *Pten*<sup>+/-</sup>, and *T-ETV1*; *Pten*<sup>+/-</sup> males in which PIN lesions were detected were at 13, 9.5, 6.5, and 6 mo of age, respectively. (B) IHC staining showing GFP expression (as a surrogate for ETV1 expression) in the PIN lesion of a *T-ETV1*; *Pten*<sup>+/-</sup> male (left) and ERG expression in the PIN lesions of a *T-ERG*; *Pten*<sup>+/-</sup> male (middle) and a *T-ΔErg*; *Pten*<sup>+/-</sup> male (right). Note, in all cases, GFP or ERG staining in PIN lesions (arrows) is stronger than that in normal-appearing prostate cells. Bars, 100  $\mu$ m. (C) Kaplan-Meier survival curve. All males were monitored for survival for at least 1 yr. The four *PbCre*; *T-3Mb-Erg*; *Pten*<sup>L/L</sup> males all survived to 1 yr and were euthanized for histology. The majority of *PbCre*; *Pten*<sup>L/L</sup> control males were still alive even after 15 mo. Log-rank test: (\*\*)  $P = 0.0013$  for *PbCre*; *T-ETV1*; *Pten*<sup>L/L</sup> in relation to *PbCre*; *Pten*<sup>L/L</sup> controls. (D) Cancer phenotypes in *PbCre*; *Pten*<sup>L/L</sup> males with or without ETS fusions. (Panel a) Gross appearance of prostates from a 10-mo-old *PbCre*; *T-ETV1*; *Pten*<sup>L/L</sup> male showing large tumor and prostatic cyst (right) and a 13-mo-old *PbCre*; *Pten*<sup>L/L</sup> control male (left). (Panel b) Typical localized prostate cancer seen in a control *PbCre*; *Pten*<sup>L/L</sup> male. (Panel c) Invasive prostate adenocarcinoma seen in a *PbCre*; *T-ETV1*; *Pten*<sup>L/L</sup> male. (Panel d) GFP staining (as a surrogate for ETV1 expression) in invasive prostate adenocarcinoma cells (brown) in a *PbCre*; *T-ETV1*; *Pten*<sup>L/L</sup> male (a magnified view of GFP<sup>+</sup> invasive prostate cancer cells is shown in the inset). (Panel e) Invasive prostate adenocarcinoma cells (arrow) detected in an aged *PbCre*; *T-3Mb-Erg*; *Pten*<sup>L/L</sup> male. (Panel f) IHC staining of Erg (brown) revealing Erg<sup>+</sup> and Erg<sup>-</sup> invasive prostate tumor cells (arrows; from the same male as in panel e) within the same section. Bars, 200  $\mu$ m.

initially relatively weak and heterogeneous (Fig. 1C). Thus, overexpression of ETV1 or ERG correlates with the observed localized premalignant phenotype.

To test cooperation of *Tmprss2-ETS* with total loss of *Pten*, we used *Pb-Cre* (Wu et al. 2001) to inactivate a conditional knockout allele of *Pten* (Lesche et al. 2002) and generated *Pb-Cre*; *T-3Mb-Erg*; *Pten*<sup>L/L</sup> males and *Pb-Cre*; *T-ETV1*; *Pten*<sup>L/L</sup> males. Prostate cancer development in these models was tracked by pAKT expression (Supplemental Fig. S4). Under our housing and genetic background (mixed C57/BL6-129), *Pb-Cre*; *Pten*<sup>L/L</sup> males developed localized PIN lesions that slowly progressed to prostate

adenocarcinomas. In contrast, the *Pten* loss-driven prostate cancer phenotype in *Pb-Cre*; *T-ETV1*; *Pten*<sup>L/L</sup> males was markedly enhanced. The majority of *Pb-Cre*; *T-ETV1*; *Pten*<sup>L/L</sup> males died before 1 yr of age, possibly due in part to large prostatic cyst formation (Supplemental Fig. S5). In contrast, the majority of *Pb-Cre*; *Pten*<sup>L/L</sup> and *Pb-Cre*; *T-3Mb-Erg*; *Pten*<sup>L/L</sup> males survived to at least 1 yr of age (Fig. 2C). On histology, we observed aggressive GFP<sup>+</sup> (from the *T-ETV1* allele) prostate adenocarcinoma cells invading into stroma in *Pb-Cre*; *T-ETV1*; *Pten*<sup>L/L</sup> prostates (Fig. 2D, panels c,d). Prostate cancer cells metastasized locally to the urogenital area (e.g., in lymphatic vessel)



(Supplemental Fig. S6A). In addition, we noted marked stromal responses, including sarcomatous-like lesions and regions with bone-like differentiation (Supplemental Fig. S6B). Since such lesions were negative for GFP (i.e., ETV1 expression) and pAKT (Supplemental Fig. S6C), we reasoned that they may represent a desmoplastic response in the stroma due to invasive prostate cancers developing nearby, as observed in other cancers (Dvorak 1986), rather than cancers arising from an epithelial-to-mesenchymal transition.

In younger *Pb-Cre;T-3Mb-Erg;Pten<sup>L/L</sup>* males (4–7 mo), the prostate phenotype appeared indistinguishable from that of *Pb-Cre;Pten<sup>L/L</sup>* controls. The four oldest *Pb-Cre;T-3Mb-Erg;Pten<sup>L/L</sup>* males in this cohort all survived to 1 yr of age and were euthanized for histological examination. In these mice, we observed regions of invasive prostate adenocarcinoma not typically seen in *Pb-Cre;Pten<sup>L/L</sup>* control males at the same age in our cohort (Fig. 2D, panel e). However, we noted that whereas some cancer cells stained strongly for Erg expression, many were negative. In particular, we detected foci of invasive adenocarcinoma with strong Erg expression, accompanied by adjacent foci of invasive adenocarcinoma with similar histology but with little or no Erg expression (Fig. 2D, panel f). We also observed regions with invasive adenocarcinoma that were largely negative for Erg in epithelial cells (Supplemental Fig S7). The dynamic expression pattern suggests that Erg expression is up-regulated and selected for in PIN lesions under the *Pten<sup>+/-</sup>* background but may not be strictly needed in invasive cancers under the total *Pten* loss background. In contrast, ETV1 expression appeared consistently homogeneous in invasive cancer cells, suggesting that its overexpression is required for cooperation with total *Pten* loss for the development of invasive adenocarcinomas (Fig. 2D, panel d).

In summary, we found that while both *Tmprss2-ETV1* and *Tmprss2-ERG* cooperate with loss of a single copy of *Pten* in the development of localized prostate cancer, only *Tmprss2-ETV1* appears to cooperate with full loss of *Pten*, leading to invasive prostate adenocarcinoma and decreased survival.

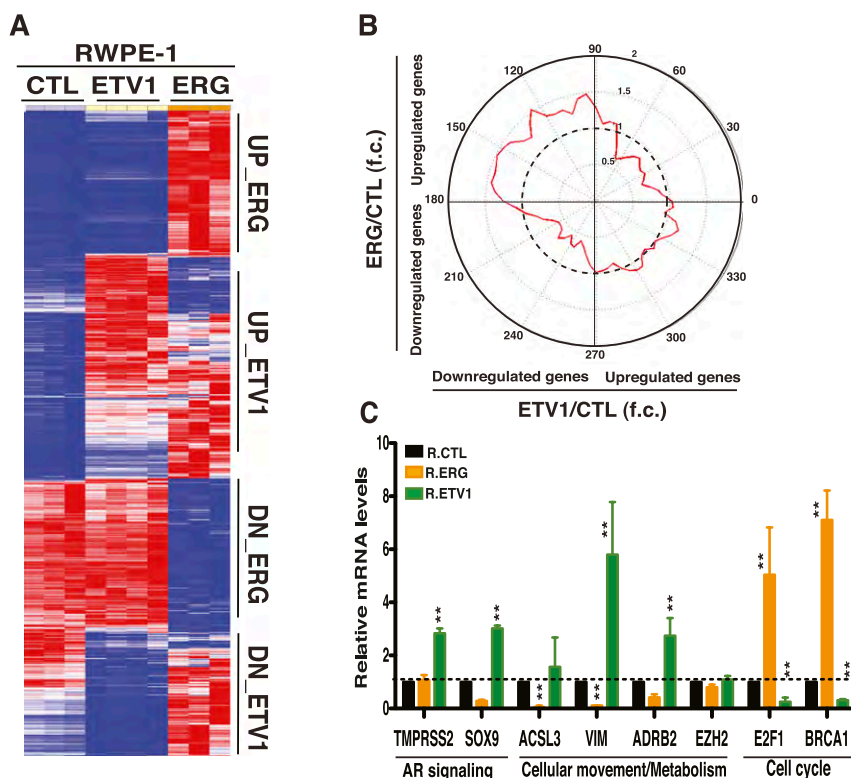
#### *ERG and ETV1 regulate distinct programs in immortalized nontumorigenic prostate cells*

The genetically engineered knock-in mice distinguished ETV1 from ERG in supporting invasive adenocarcinoma. To gain mechanistic insights into this difference, we performed an integrated genomic analysis to identify their respective target genes. First, we ectopically expressed ETV1 or ERG in immortalized human nontumorigenic prostate epithelial cells, RWPE-1 cells. Thus, RWPE-1 cells were engineered to express full-length *ERG* or *ETV1* cDNA carrying a substrate tag that permits in vivo biotinylation by coexpressed *Escherichia coli* biotin ligase BirA (bioERG and bioETV1) (Supplemental Fig. S8A,B). RWPE-1 cells overexpressing bioERG (R.ERG) or bioETV1 (R.ETV1) proliferated normally compared with controls (Supplemental Fig. S8C). Microarray expression profiling data strongly overlapped with those

previously reported (ERG [Gupta et al. 2010] and ETV1 [Tomlins et al. 2007] in RWPE-1 cells [Supplemental Fig. S8D,E]) and yielded two findings. First, a set of genes was uniquely regulated by either ERG or ETV1. Second, both factors also regulated a common set of genes but in a largely opposing fashion (Fig. 3A). This latter observation is depicted by the quantitative two-dimensional comparison of the ERG and ETV1 data sets, which illustrates a statistically significant correlation between ERG-driven up-regulated genes and ETV1-driven down-regulated genes and vice versa (Fig. 3B). Gene ontology (GO) and Ingenuity pathway analysis (IPA) implicated genes up-regulated upon ERG expression and down-regulated upon ETV1 expression that are associated with cell proliferation. In contrast, genes down-regulated on ERG expression and up-regulated by ETV1 are correlated with cell motility and lipid metabolism (Supplemental Fig. S9). As revealed by quantitative RT-PCR of select common genes, ETV1 expression induced up-regulation of genes involved in AR signaling (*TMPRSS2* and *SOX9*) or invasion and lipid metabolism (*VIMENTIN*, *ADRB2*, and *ACSL3*) as well as down-regulation of cell cycle genes (*E2F1* and *BRCA1*) (Fig. 3C). In contrast, these genes exhibited largely an opposite expression pattern in ERG-expressing RWPE-1 cells (Fig. 3C). Thus, these data point to distinct regulatory programs driven by ERG and ETV1 in non-tumorigenic prostate cells.

#### *ERG and ETV1 have shared and distinct chromatin targets*

To interrogate further similarities and differences of ERG and ETV1 transcriptional programs, we performed ChIP (chromatin immunoprecipitation)-on-chip analysis. We identified ERG ChIP targets in VCaP cells (harboring *TMPRSS2-ERG*) by an anti-ERG antibody. As a ChIP-quality antibody for ETV1 is not available, we used the bio-ChIP approach (Supplemental Fig. S10A; de Boer et al. 2003; Wang et al. 2006; Kim et al. 2008) on LNCaP cells (harboring *ETV1* fusions) ectopically expressing bioETV1 and the *E. coli* biotin ligase BirA (Supplemental Fig. S10B). On comparison of ERG and ETV1 ChIP targets (Supplemental Table S1), we identified three subsets: ERG–ETV1 common targets, ERG-only targets, and ETV1-only targets (Fig. 4A), which are consistent with reported ChIP-seq (ChIP combined with deep sequencing) data in RWPE-1 cells (Hollenhorst et al. 2011). Bound regions for each factor were typically in close proximity to the transcription start sites (TSSs) (Supplemental Fig. S10C,D). We confirmed that ERG–ETV1 common target regions were occupied by both ERG and ETV1. As anticipated, ETS-binding motifs are the most statistically significant enriched motifs within the predicted targets (Fig. 4B). Further analysis of the defined target subsets revealed clear differences of motif enrichment at common versus unique targets (Fig. 4B; Supplemental Fig. S10E), consistent with expression profiling data indicating distinct biological processes regulated by ERG and ETV1 in prostate cells (Supplemental Figs. S9A,B, S10F).



**Figure 3.** ERG and ETV1 regulate a common program in immortalized nontumorigenic RWPE-1 prostate cells but in an opposing fashion. (A) Expression profiling of ERG-overexpressing (R.ERG) and ETV1-overexpressing (R.ETV1) RWPE-1 cells compared with BirA-expressing controls (CTL). Heat map generated by hierarchical clustering and by applying Pearson correlation and the complete linkage rule. The heat map shows differentially expressed genes (fold change, >1.5; false discovery rate [FDR], <0.05). (Red) Highest expression; (blue) lowest expression. (B) Bidimensional plot comparing expression profiles of genes differentially expressed (fold change, >1.5) in R.ERG versus R.CTL and in R.ETV1 versus R.CTL RWPE-1 cells. The red line represents the distribution of genes. The dotted line corresponds to a gene density fold change of 1. (C) RT-PCR analysis of select genes associated with prostate cancer pathways upon ERG or ETV1 overexpression in RWPE-1 cells.  $n = 3$  per group. Error bars, SEM; t-test: (\*)  $P < 0.01$ . If no  $P$ -value is indicated,  $P > 0.05$ .

Interestingly, IPA analysis indicated that nuclear receptor signaling pathways, including those associated with estrogen, androgen, and glucocorticoid receptor signaling, were significantly enriched in ERG–ETV1 common targets (Fig. 4C). In contrast, the ERG-only subset correlated with the cell cycle network. Intriguingly, the lipid metabolism biological network as well as the Oncostatin M and IL-3 signaling pathways, which have been correlated with increased cell motility and invasiveness (Dentelli et al. 1999; Holzer et al. 2004), were enriched in the ETV1-only subset (Fig. 4C). Taken together, our combined gene expression and ChIP-on-chip analyses argue that ERG and ETV1 control distinct transcriptional programs in prostate cells.

#### *ERG and ETV1 interact differentially with the AR signaling pathway*

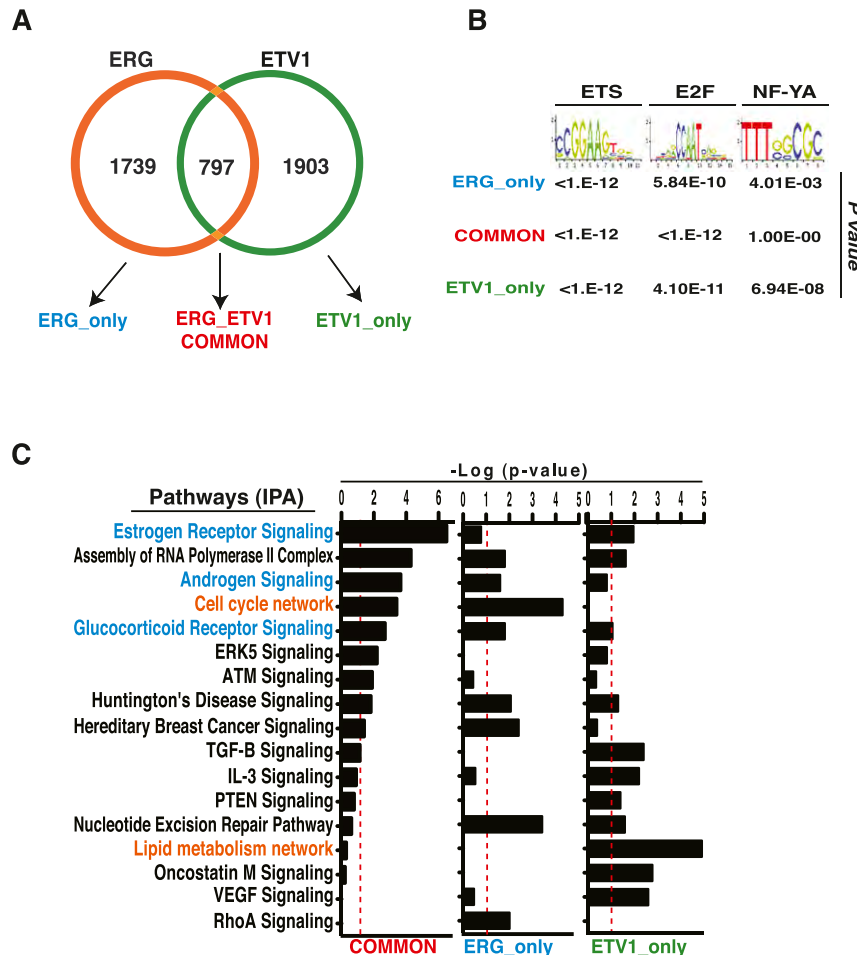
The AR pathway is a critical driver of tumorigenic prostate development in both androgen-dependent (AD) and castration-resistant stages (Wang et al. 2009). Our data suggest that genes associated with AR signaling belong to the ERG–ETV1 common target category (Fig. 4C; Supplemental Fig. S11A). To address potential cross-talk of ERG and ETV1 common targets with the AD program, we defined an improved androgen-driven signature from AD VCaP and LNCaP cell lines that mitigates differences among diverse expression-based gene sets (Supplemental Fig. S12). Gene set enrichment analysis (GSEA) (Subramanian et al. 2005) revealed that this signature was significantly depleted after *ETV1* knockdown in LNCaP cells (Fig. 5A) but enhanced in VCaP cells upon

*ERG* silencing (Fig. 5B). This finding was confirmed by quantitative RT-PCR for select AR-driven genes in LNCaP and VCaP cells (Figure 5C,D). ChIP analysis further demonstrated that, while androgen stimulated AR and ETV1 binding to the enhancers and promoters of two established AR targets, *PSA* and *TMPRSS2*, androgen decreased ERG occupancy to these AR target genes (Supplemental Fig. S11B,C). These data indicate that ETV1 cooperates with activation of AR signaling, while ERG negatively modulates the AR transcriptional program.

To determine whether ERG and ETV1 also regulate AR signaling differentially in vivo, we examined GFP expression in *T-ERG* and *T-ETV1* males, as the reporter serves as a surrogate for the in vivo activity of the *Tmprss2* promoter in these identically engineered mice. GFP expression was readily detected in *T-ETV1* prostates but barely detected in *T-ERG* prostates (Fig. 5E). In contrast, GFP expression was detected in estrogen receptor-positive mammary epithelial cells (Sleeman et al. 2007) of both *T-ERG* and *T-ETV1* females (Supplemental Fig. S13). These data indicate that *Tmprss2* is indeed an estrogen and androgen dual-responsive promoter and that its promoter activity is down-regulated in *T-ERG* prostate cells.

To ascertain whether elevated AR signaling up-regulates the *Tmprss2* promoter activity in vivo, we bred *T-ERG* and *T-ETV1* mice to transgenic mice that express a mutated version of AR (E231G) expressed from the *Pb* promoter (*Pb-AR*) (Han et al. 2005). In *Pb-AR*; *T-ERG* double-transgenic males, we detected a strong GFP signal in the prostate (Fig. 5E). The *Pb-AR* transgene is most

## ETV1 promotes aggressive prostate cancer phenotype



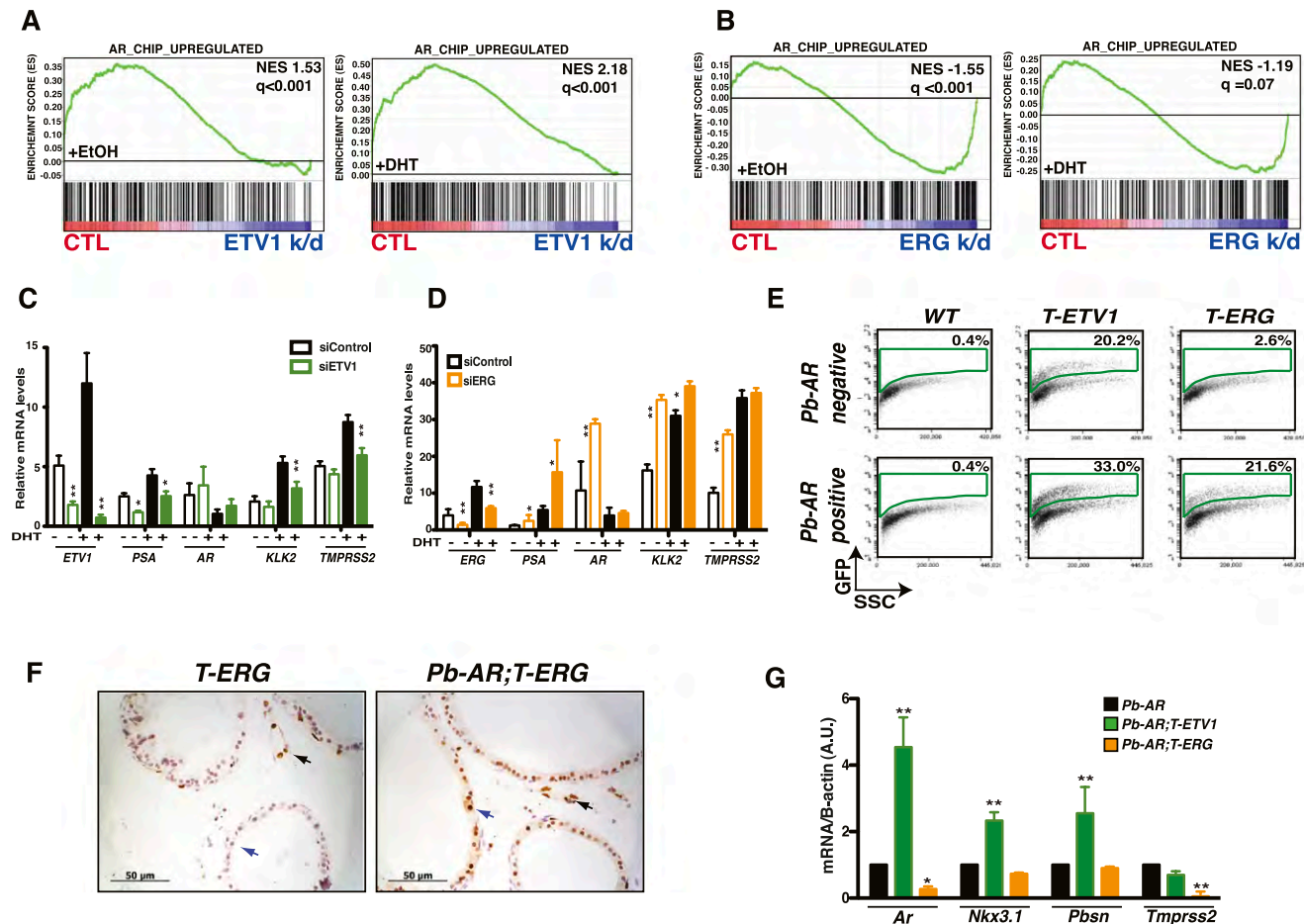
**Figure 4.** ERG and ETV1 drive specific transcriptional programs. (A) Venn diagram of targets occupied by ERG and ETV1. The intensity of binding at each probe was calculated by model-based analysis of tiling array (MAT) (Johnson et al. 2006). MAT scores were then normalized by quantile-quantile normalization (Bolstad et al. 2003) between ETV1 and ERG ChIP-on-chip experiments. Target loci were defined as the peaks associated with  $P$ -value  $< 10^{-4}$ . (B) Enrichment of ETS-binding motifs and other indicated motifs in all ChIP target subsets. The Fisher exact test was applied. (C) IPA analysis of ChIP-defined target gene sets implicating common target genes in nuclear receptor signaling pathways and ETV1 unique targets in lipid metabolism network. The significance of enrichment of each gene set is shown as  $-\log(P\text{-value})$ .

active in the ventral prostate lobes (Han et al. 2005). In accord with this, we observed stronger staining for ERG in the ventral lobes of *Pb-AR*; *T-ERG* prostates, compared with barely detectable ERG staining in *T-ERG* prostates (Fig. 5F). Furthermore, we measured expression levels of select AR targets in mouse prostates. In the *Pb-AR* background, most AR targets were down-regulated in *T-ERG* males, whereas AR targets were typically up-regulated in *T-ETV1* males, thus illustrating the opposite regulation of AR signaling by ERG and ETV1 in vivo (Fig. 5G). Despite elevated AR signaling, prostates of *Pb-AR*-alone as well as those of *Pb-AR*; *T-ERG* and *Pb-AR*; *T-ETV1* males appeared largely normal. Taken together, human prostate cancer cell and mouse model data indicate that differential regulation of the AR pathway by ETV1 and ERG occurs not only in vitro, but, importantly, also under the physiological setting.

#### ETV1 directs androgen metabolism in prostate epithelial cells

In addition to the opposing regulation of common targets by ETV1 and ERG, we hypothesized that unique targets controlled by ETV1 might contribute to the aggressive phenotype seen in association with ETV1 expression. To

gain mechanistic insights into programs selectively regulated by ETV1 we sorted GFP<sup>+</sup> (thus, ETV1-expressing) prostate luminal cells from *T-ETV1* knock-in males and compared them with luminal cells from wild-type prostates by microarray expression profiling (Fig. 6A,B). We confirmed the luminal cell expression pattern in both sorted samples (Supplemental Fig. S14). By GSEA, we identified several cancer-associated metabolic pathways that were enriched in *T-ETV1* luminal cells (Supplemental Fig. S15A). Of note, cholesterol and steroid biosynthesis pathways, both of which are strongly related to prostate tumorigenesis (Twiddy et al. 2010; Zadra et al. 2010), were most highly enriched (Fig. 6C,D). On analysis of a patient cohort with CRPCs metastatic to bone (Stanbrough et al. 2006), we observed that genes associated with the steroid hormone biosynthesis pathway and androgen and estrogen metabolism are significantly enriched in tumors with higher ETV1 expression (Supplemental Fig. S15A). *HSD17B7*, a gene shared by steroid biosynthesis and steroid hormone biosynthesis pathways, was up-regulated in both *T-ETV1* luminal cells and ETV1-high bone metastases (Supplemental Fig. S15B). *HSD17B7* as well as other HSD17B enzyme genes (*HSD17B4* and *HSD17B10*) are ChIP targets of ETV1 in prostate cancer cells and are components of the lipid metabolism



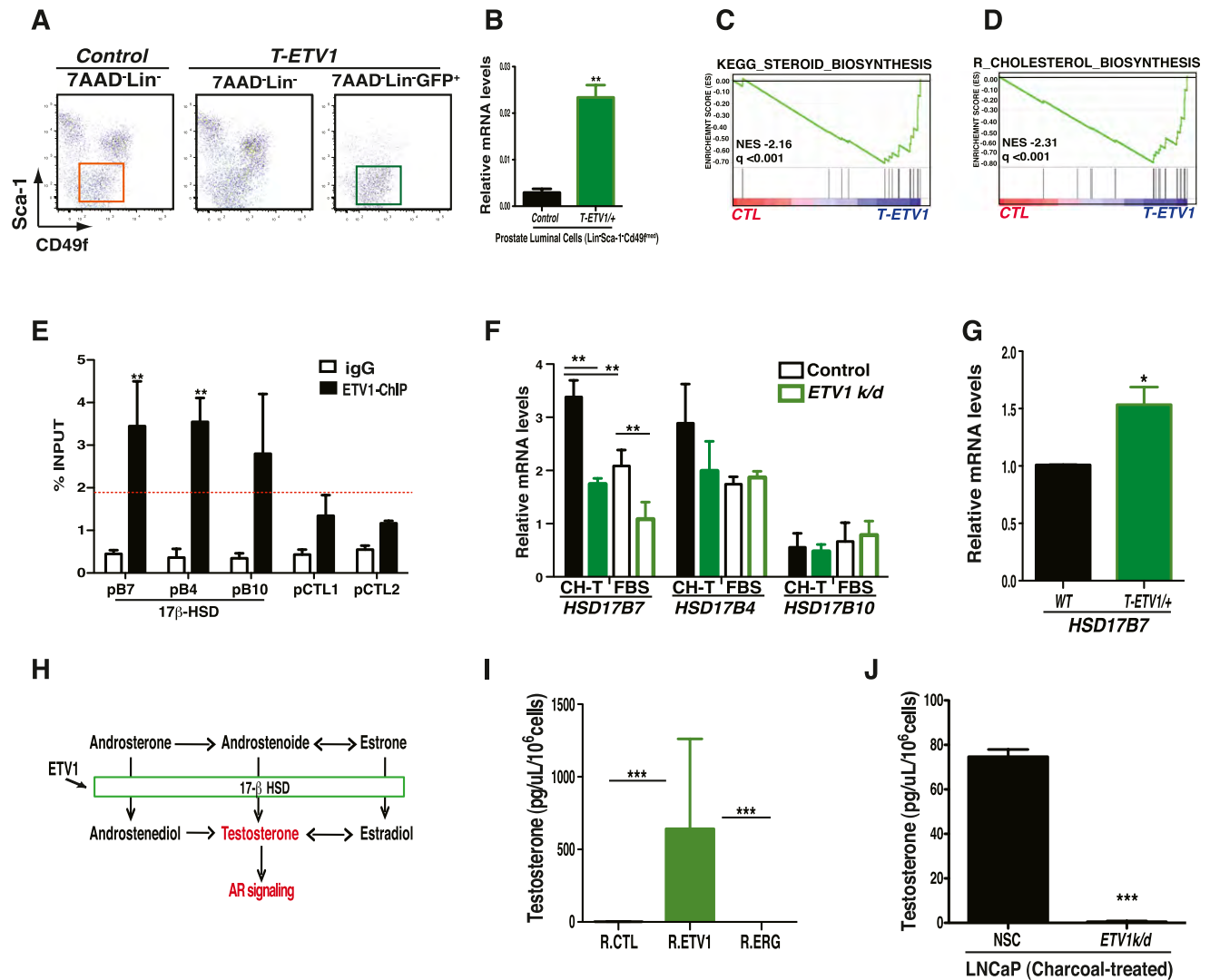
**Figure 5.** ERG and ETV1 regulate AR signaling in an opposite manner. (A) Androgen-induced genes are depleted in *ETV1*-silenced LNCaP cells upon 16-h androgen stimulation ([left] no androgen stimulation; [right] with androgen stimulation). The androgen-induced signature was obtained from the common AR ChIP targets in LNCaP and VCaP cells that were up-regulated in them upon androgen stimulation. (B) Androgen-induced genes are significantly enriched in *ERG*-silenced VCaP cells upon 16-h androgen stimulation compared with controls ([left] no androgen stimulation; [right] with androgen stimulation). (C,D) *ETV1* silencing specifically decreases expression of AR-associated genes (C), whereas *ERG* silencing increases their expression (D). Mean,  $n = 3$ ; error bars, SEM;  $t$ -test: (\*)  $P < 0.05$ ; (\*\*)  $P < 0.01$ . If no  $P$ -value is indicated,  $P > 0.05$ . (E) Flow cytometry analysis demonstrating robust GFP<sup>+</sup> population in the *T-ETV1* prostates but not in the *T-ERG* prostates. However, in the presence of the *Pb-AR* transgene, GFP expression can be readily detected in *Pb-AR;T-ERG* prostates; in addition, GFP expression in *Pb-AR;T-ETV1* prostates appear to be further elevated. (F) IHC staining showing weak ERG staining in the ventral lobe of a *T-ERG* knock-in male (blue arrow; compared with strong Erg staining in the endothelial cells [black arrow]) but much stronger ERG staining in the ventral lobe of a *Pb-AR;T-ERG* male (blue arrow; almost comparable with ERG staining in endothelial cells in the same section [black arrow]). Bars, 50  $\mu$ m. (G) Real-time PCR quantification showing up-regulation of most AR target genes in *Pb-AR;T-ETV1* prostates and slight down-regulation of them in *Pb-AR;T-ERG* prostates in relation to those of *Pb-AR*-alone prostates. Mean,  $n = 3$ ; error bars, SEM;  $t$ -test: (\*)  $P < 0.05$ , (\*\*)  $P < 0.01$ . If no  $P$ -value is indicated,  $P > 0.05$ .

network enriched in *ETV1*-only ChIP targets (Fig. 4C; Supplemental Fig. S15C). By independent ChIP analysis, we validated *ETV1*, but not *ERG*, binding to the *HSD17B7* and *HSD17B4* promoters (Fig. 6E; Supplemental Fig. S15D). Expression of *HSD17B7* in LNCaP cells was reduced upon *ETV1* depletion (Fig. 6F). In *ETV1*-over-expressing RWPE-1 cells, *HSD17B7* expression trended upward (although not statistically significant) (Supplemental Fig. S16A). We also confirmed higher *Hsd17b7* expression in lineage-depleted *T-ETV1* prostate cells (Fig. 6G). Since *HSD17B7* is critical in converting less active forms of estrogen and androgen to more active forms (Fig. 6H; Krazeisen et al. 1999), and *Tmprss2* is an androgen

and estrogen dual-responsive gene, we reasoned that up-regulation of the steroid biosynthesis pathway by *ETV1* may provide prostate cells with an intrinsic source of steroids. If this supposition were correct, such cells might be intrinsically castration-resistant. To test this prediction, *T-ETV1* knock-in males as well as *T-ERG* and wild-type control males were castrated. We observed that almost half of prostate cells from the castrated *T-ETV1* mice were GFP<sup>+</sup>. In addition, we also detected a small population of GFP<sup>low</sup> prostate cells from the castrated *T-ERG* males (Supplemental Fig. S16B), consistent with a recent study and possibly reflecting the existence of a subpopulation of *Tmprss2*<sup>+</sup> prostate cells that are intrinsically castration-



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**Figure 6.** ETV1 regulates steroid metabolism in prostate cells. (A, right plot) Flow cytometry profiles and gating strategies showing GFP<sup>+</sup> prostate luminal cells (Lin<sup>-</sup>Sca-1<sup>-</sup>CD49f<sup>med</sup>) sorted from *T-ETV1* knock-in males used for microarray analysis. (Left plot) The Lin<sup>-</sup>Sca-1<sup>-</sup>CD49f<sup>med</sup> prostate luminal cells sorted from wild-type (WT) control males were used as the control. (B) Real-time PCR quantification confirming ectopic *ETV1* expression in sorted GFP<sup>+</sup> prostate luminal cells from *T-ETV1* knock-in mice (mean,  $n = 3$  samples per group; error bars, SEM). (C,D) Steroid and cholesterol biosynthesis pathways are the top pathways significantly enriched in *T-ETV1* knock-in prostate luminal cells compared with controls. Note that a critical enzyme in the steroid biosynthesis pathway, *HSD17B7*, is also a key enzyme in the steroid hormone biosynthesis pathway, which is enriched in metastatic prostate cancers (Supplemental Fig. S15). (E) ChIP-PCR validation of ETV1 binding to *HSD17B7* (pB7), *HSD17B4* (pB4), and *HSD17B10* (pB10) promoters (mean,  $n = 5$ ; error bars, SEM). (pCTL1 and pCTL2) Nonspecific promoter control regions. (F) Only *HSD17B7* levels significantly decreased upon knockdown of ETV1 (k/d) (mean,  $n = 3$ , error bars, SEM) under both the androgen-deprivation condition ([CH-T] charcoal-treated) and the regular condition in the presence of serum (FBS). Conversely, *HSD17B7* expression increased upon ETV1 overexpression in RPWE-1 cells (Supplemental Fig. S16A).  $t$ -test: (\*\*)  $P < 0.01$ . If no  $P$ -value is indicated,  $P > 0.05$ . (G) *T-ETV1* knock-in prostate cells exhibit increased *Hsd17b7* expression levels compared with wild-type controls (mean,  $n = 3$ ; error bars, SEM).  $t$ -test: (\*)  $P$ -value  $< 0.05$ . (H) Schematic diagram showing the key role of 17- $\beta$  HSD enzymes, including *HSD17B7*, in converting androgen and estrogen from their less active forms to active forms. (I) ETV1 overexpression in RWPE-1 cells promoted the elevation of the endogenous testosterone level, while no changes were observed upon ERG overexpression (mean,  $n = 4$ ; error bars, SEM). Testosterone levels per  $10^6$  cells (R.ETV1 mean = 642.16 pg/ $\mu$ L; R.ERG mean = 0.49 pg/ $\mu$ L; R.CTL mean = 1.89 pg/ $\mu$ L).  $t$ -test: (\*\*\*)  $P < 0.001$ . (J) Testosterone levels were reduced in androgen-deprived (charcoal-treated) LNCaP cells upon stable ETV1 silencing (k/d) as compared with controls (mean,  $n = 3$ ; error bars, SEM). (NSC) Nonsilencing shRNA control. Testosterone levels per  $10^6$  cells (NSC mean = 74.69 pg/ $\mu$ L; R.ERG mean = 0.49 pg/ $\mu$ L; *ETV1k/d* mean = 0.56 pg/ $\mu$ L).  $t$ -test: (\*\*\*)  $P < 0.001$ .

resistant (Casey et al. 2012). As expected, the castration-resistant GFP<sup>+</sup> cells in *T-ETV1* males exhibited higher *Hsd17b7* levels than controls (Supplemental Fig. S16C).

To determine whether increased ETV1 expression enhances steroid production, we directly measured by liquid chromatography/mass spectrometry (LC/MS) the levels

of endogenous testosterone in control, ETV1-expressing, and ERG-expressing human RWPE-1 cells. Remarkably, ETV1-expressing RWPE-1 cells showed much higher levels (>300-fold higher) of endogenous testosterone compared with ERG-expressing and control RWPE-1 cells (Fig. 6I; Supplemental Fig. S17A,B). Conversely, *ETV1* knockdown in LNCaP cells reduced testosterone production under conditions of androgen deprivation (Fig. 6J). To further characterize this observation, we quantified the intraprostatic levels of testosterone in wild-type, *T-ETV1*, and *T-ERG* mice under noncastrated and castrated conditions. As expected, noncastrated mice showed very low concentrations of testosterone in their prostate cells. Interestingly, castrated *T-ETV1* mice indeed exhibited higher testosterone levels than those of castrated wild-type and *T-ERG* prostate samples (Supplemental Fig. S17C). These results indicate that ETV1 expression directly regulates androgen production in prostate cells.

#### *ETV1-only gene sets associate with an aggressive phenotype in patients*

Data to this point suggest that ETV1 and its unique oncogenic program contribute to invasive prostate cancer. To relate these findings to patients, we analyzed data from a patient cohort that includes 22 primary localized and 29 metastatic samples, of which ~50% carried the *TMPRSS2-ERG* fusion (ETV1 rearrangement status not characterized) (Stanbrough et al. 2006). In this cohort, samples exhibiting higher *ERG* expression correlated with localized tumors, whereas high *ETV1* expression enriched for metastases (Fig. 7A). We repeated the analysis with an independent cohort of 150 prostate tumor samples from patients at Memorial Sloan-Kettering Cancer Center (MSKCC) (Taylor et al. 2010). A correlation between high *ETV1* expression and metastases and the presence of more localized prostate tumors in the high *ERG* expression group were confirmed in this second cohort (Fig. 7B). Samples with high *ERG* or high *ETV1* expression did not overlap, consistent with a strong tendency to mutual exclusivity in both primary and metastatic samples (Supplemental Fig. S18A,B). We next interrogated whether *ERG* and *ETV1* cooperate similarly with *PTEN* deletion in the MSKCC cohort by analyzing the outcome of patients carrying deletion of *PTEN* and overexpression of *ERG* or *ETV1*. High *ERG* expression with *PTEN* loss failed to correlate with the worse outcome (Fig. 7C). In contrast, high *ETV1* expression cooperated with *PTEN* loss, as shown by much poorer disease-free survival (Fig. 7D). These data are consistent with a previous report correlating greater disease recurrence with high *ETV1* levels (Shin et al. 2009).

We next evaluated for the first time whether ERG- and ETV1-specific gene signatures serve as tumor biomarkers or as a predictor of aggressive behavior. We analyzed ERG and ETV1 signatures defined in our analysis of ERG and ETV1 ChIP and expression profiling data in the Swedish watchful waiting and MSKCC cohorts (Setlur et al. 2008; Taylor et al. 2010), including 362 localized prostate cancer samples and 150 patients with localized and

metastatic prostate cancer, respectively. Of note, ETV1-specific signatures, comprised of genes directly bound by ETV1 and up-regulated upon ETV1 expression, are associated with a high Gleason score (>7) in both cohorts and with lethality in the MSKCC cohort, again highlighting a correlation between ETV1 expression and a worse disease prognosis (Fig. 7E; Supplemental Fig. S18C). Taken together, data from three independent patient cohorts concur in validating that ETV1 drives a transcriptional program in prostate cells that is distinct from that of ERG. Moreover, our data suggest for the first time that the ETV1-driven program dictates a poorer outcome in patients with prostate cancer.

## Discussion

Our multidisciplinary studies reveal distinct transcription programs regulated by ERG and ETV1 in prostate cells. In particular, we show that they control overlapping gene targets but largely in an opposing fashion; they also control unique targets and pathways. Overall, the networks regulated by ERG are associated with cell cycle and DNA replication, whereas those controlled by ETV1 are related to synthesis of lipids and other metabolic pathways. These networks are distinct and contribute to different pathogenic consequences. These conclusions are validated by findings in novel knock-in mouse models and by patient outcome analysis.

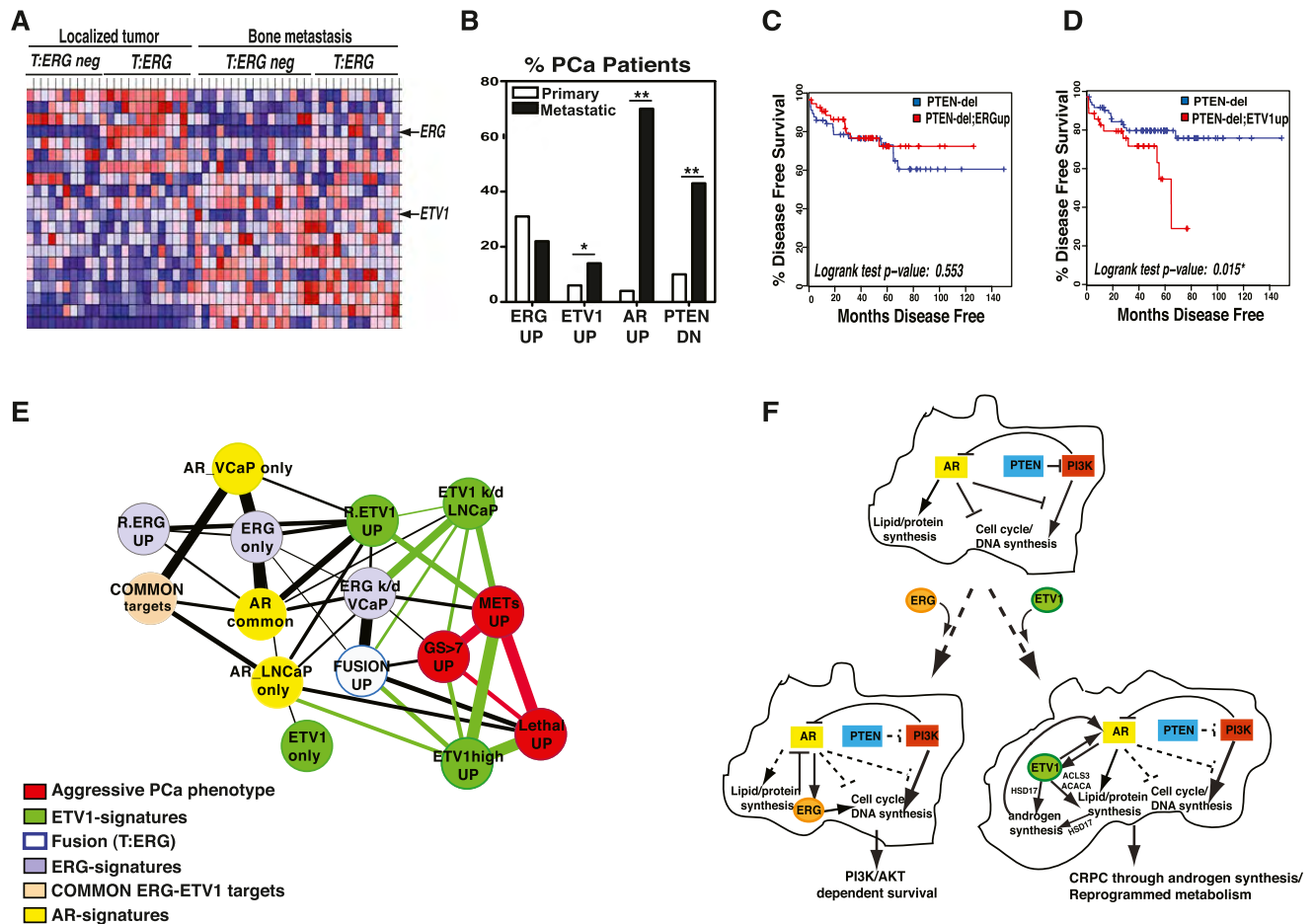
#### *AR signaling is a common pathway regulated by ERG and ETV1 but in an opposite manner*

AR signaling is central to prostate development and tumorigenesis. Indeed, AR has recently been implicated in double-strand breaks that favor the formation of translocations involving androgen-driven promoters and ETS family members (Lin et al. 2009; Haffner et al. 2010). We observed a complex relationship between ERG- or ETV1-regulated networks and AR signaling. In agreement with prior findings, we observed negative regulation of AR signaling by ERG (Yu et al. 2010). In contrast, ETV1 cooperates with AR signaling by favoring activation of the AR transcriptional program (Fig. 5). Upon androgen stimulation, ETV1 recruitment to the established *PSA* and *TMPRSS2* regulatory elements correlates with AR binding, suggesting coordinate regulation of androgen-driven genes by ETV1 and AR.

We validated divergent regulation of AR signaling by ERG and ETV1 in vivo in knock-in mice. As *Tmprss2* is an AR target, the transcriptional output from the *Tmprss2* promoter serves as a reporter for AR activity. In *T-ERG* knock-in mice, ERG expression appears to down-regulate AR target genes, including *Tmprss2*, which would lead to down-regulation of its own expression, thus forming a negative regulatory loop, consistent with prior findings (Yu et al. 2010). In contrast, in *T-ETV1* knock-in males, ETV1 expression positively cooperated with AR signaling, leading to further enhancement of expression of AR targets, including *Tmprss2*, which would then support robust expression of ETV1 and the GFP reporter, reflecting a



## ETV1 promotes aggressive prostate cancer phenotype



**Figure 7.** ETV1, rather than ERG, expression and the program it drives are associated with advanced prostate cancer in multiple patient cohorts. (A) Heat map showing ERG and ETV1 expression pattern in localized and bone metastatic prostate cancer samples using the Beth-Israel (BI) cohort data set (Stanbrough et al. 2006). Heat map generated by hierarchical clustering and by applying Pearson correlation and the complete linkage rule. Heat map showing differentially expressed select genes (fold change,  $>2$ ; FDR,  $<0.05$ ). (B) Graph showing ERG and ETV1 expression along prostate cancer progression from localized to metastatic samples in the MSKCC cohort (Taylor et al. 2010). The graph reveals that the number of patients carrying ETV1 overexpression (fold change,  $>3$ ), PTEN deletion, and AR alterations (amplification and expression fold change,  $>3$ ) increased in metastatic samples compared with localized prostate tumors, while patients carrying high levels of ERG (fold change,  $>3$ ) did not increase over time (also in Supplemental Fig. S18B).  $t$ -test: (\*)  $P$ -value  $< 0.05$ ; (\*\*)  $P$ -value  $< 0.01$ . If no  $P$ -value is indicated,  $P > 0.05$ . (C) Disease-free survival plot showing that among all patients in the MSKCC cohort with PTEN deletion ( $n = 21$ ), those with ERG overexpression ( $n = 4$ ) exhibited no survival difference from the rest of patient with PTEN deletion.  $P = 0.553$  by log-rank test. (D) Disease-free survival plot showing that among all patients in the MSKCC cohort with PTEN deletion ( $n = 21$ ), those with ETV1 overexpression ( $n = 8$ ) exhibited much worse survival compared with remaining patients with PTEN deletion. (\*)  $P = 0.015$  by log-rank test. (E) Correlation between ERG- and ETV1-associated gene sets with patient prognosis. Overlap between genes enriched in patient samples associated with indolent or aggressive prostate cancer from the MSKCC cohort (Taylor et al. 2010) and ETV1 or ERG gene sets defined in Figures 3 and 4. ETV1-associated genes are enriched in patients with a higher Gleason score in the Swedish cohort (also in Supplemental Fig. S18C; Setlur et al. 2008). "UP" represents those genes up-regulated in the shown category with a fold change of  $>1.5$ . The significance of overlap of these gene sets was calculated by the Fisher exact test and visualized as connecting line width (cutoff,  $P = 0.01$ ). (Red) Aggressive prostate cancer-associated; (green) ETV1-associated gene sets; (blue) *TMPRSS2-ERG* fusion-associated gene sets; (purple) ERG signature-associated gene sets; (yellow) AR-associated gene sets; (orange) common targets of ERG and ETV1. (F) Model illustrating the differential contribution by ERG and ETV1 to prostate tumorigenesis under the PTEN loss background. See the text for details.

positive regulatory loop. These mechanistic differences account for the striking difference in GFP intensity in the two knock-in strains, which were identically engineered (Fig. 5E). Of note, the negative loop in the *T-ERG* knock-in is overridden by elevated AR signaling in vivo. Indeed, the only consistent association between the *TMPRSS2-ERG*

fusion and clinical parameters identified thus far is the association of this fusion (in particular when ERG expression was measured by IHC) with a high level of AR signaling (Minner et al. 2011; Hoogland et al. 2012). Last, in human prostate tumors with ETV1 fusions, several 5' fusion partners other than *TMPRSS2* are also involved.

Since the majority of these 5' fusion partners are also androgen-responsive genes (Tomlins et al. 2007; Attard et al. 2008b), we reason that a similar positive regulatory loop may be operative in such tumors to enhance AR signaling and *ETV1* expression. In addition, AR has been reported to regulate the endogenous *ETV1* locus as well (Cai et al. 2007), suggesting a positive feedback between both the endogenous and rearranged *ETV1*, thus predisposing affected prostate cells for prostate cancer development.

*ETV1, but not ERG, reprograms metabolic pathways in normal prostate epithelial cells*

Among the unique genes up-regulated by *ETV1*, those encoding for enzymes involved in cholesterol and steroid biosynthesis and in cancer-associated metabolic pathways (e.g., glycolysis) (Supplemental Fig. S15A) are of particular interest. In particular, we were able to demonstrate increased production of endogenous testosterone upon *ETV1* expression in both human prostate cells and *T-ETV1* knock-in mice (upon castration) (Fig. 6I,J; Supplemental Fig. S17C). Due to the increased testosterone level, we anticipated observing higher levels of androstenedione or dihydrotestosterone as well; however, none were detected, possibly due to technical limitations. However, decreased levels of estrone were detected in *ETV1*-expressing RWPE-1 cells, probably due to the promotion of testosterone synthesis (Supplemental Fig. S17B).

Although *ETV1*-expressing prostate cells appear ostensibly normal (Fig. 1E), their metabolic programs resemble those of cancer cells. Increased aerobic glycolysis has been observed only in advanced disease, whereas increased sterol and protein synthesis are common features of both primary and advanced prostate cancer (Swinnen et al. 2000; Rossi et al. 2003; Ettinger 2004; Bauer et al. 2005). In particular, activation of lipid metabolism has been described in most localized and metastatic prostate tumors, underscoring its potential role in prostate cancer progression (Twiddy et al. 2010; Zadra et al. 2010). Arachidonic acid metabolism and Toll-like receptor signaling inflammatory pathways (De Marzo et al. 2007) are enriched in both CRPCs with higher *ETV1* expression and *T-ETV1* mouse prostate cells, correlating with the extended areas of inflammation observed in *T-ETV1* prostates (Fig. 1E). Thus, we speculate that this unique *ETV1*-controlled program, in concert with positive cooperation with AR signaling, may reprogram prostate cells for malignant progression in association with additional oncogenic events (Fig. 7F).

*TMPRSS2-ETV1 and TMPRSS2-ERG fusions make distinct contributions to prostate tumorigenesis*

Although both *Tmprss2-ERG* and *Tmprss2-ETV1* cooperate with loss of a single copy of *Pten* in leading to localized PIN lesions, similar to those demonstrated previously in mice overexpressing *ERG* or *ETV1* under the *Probasin* or viral promoters (Carver et al. 2009; King et al. 2009; Zong et al. 2009) or in mice expressing *ERG* from an extended human *TMPRSS2* promoter (Casey et al. 2012),

our mechanistic and animal model studies suggest they do so differently (Fig. 7F). Ectopic *ERG* expression likely represses the differentiation program of prostate cells (e.g., represses AR and AR targets and up-regulates *Ezh2* and its targets, as suggested previously) (Yu et al. 2010). Interestingly, it has been reported that *Pten* loss leads to a castration-like phenotype by suppressing androgen-responsive gene expression through modulation of AR transcriptional activity. Moreover, conditional deletion of AR further promotes proliferation of prostate cells with *Pten* loss (Mulholland et al. 2011). We speculate that *TMPRSS2-ERG* may act in a similar fashion by down-regulating AR and, consequently, promoting cell proliferation. Indeed, the *ERG* expression pattern in our murine models (Figs. 1C, 2B,D) suggests that *ERG* may be required primarily at early stages of the disease but may be not as functionally relevant at late stages. This may explain our observation that *ERG* cooperates with *Pten* haploinsufficiency (i.e., under a more sensitized *Pten*<sup>+/-</sup> background), whereas its contribution under the total *Pten* loss background appears far less. The high levels of *ERG* expression often observed in localized fusion-positive human prostate cancers may be a secondary consequence of high activity of AR signaling in such tumors in general (i.e., similar to what we observed in *Pb-AR;T-ERG* prostates) (Fig. 5E–G) rather than a critical requirement of *ERG* overexpression at this stage. In contrast, ectopic *ETV1* expression appears to enhance androgen signaling and reprogram the metabolism of prostate cells, processes critical for both early and advanced stages of the disease. Activation of the PI3K/AKT pathway drives anabolic metabolism and tumorigenesis (Ward and Thompson 2012). We propose that *TMPRSS2-ETV1* cooperates with *Pten* loss by further enhancing metabolic reprogramming, in particular, by favoring steroid biosynthesis, a pathway critical for invasive adenocarcinoma cells. The cooperation between *ETV1* and *Pten* loss is also consistent with the recent finding that combined loss of *Pten* and *COP1*, a ubiquitin ligase that negatively regulates *ETV1* levels, leads to more invasive prostate adenocarcinomas (Vitari et al. 2011).

In aged *PbCre;T-3Mb-Erg;Pten*<sup>L/L</sup> males, we also observed invasive prostate cancer (Fig. 2E). Interestingly, in such invasive cancers, we observed a mosaic pattern of *Erg* expression (Fig. 2E). Several possibilities can explain this observation. *Erg*-expressing prostate cells may send signals to *Erg*-negative cells so that high levels of *Erg* expression are not needed in all cells of the invasive cancer. Alternatively, *Erg* may not be critical for the development of advanced cancer, and another genetic or epigenetic change may contribute to advanced disease. One potential genetic change is haploinsufficiency of one or more deleted genes in the interstitial region. Interestingly, *ETS2*, a gene residing within the interstitial region, was recently proposed to be a tumor suppressor contributing to aggressive prostate cancer cases carrying *TMPRSS2-ERG* fusions with deletion (Grasso et al. 2012). Whether haploinsufficiency of the deleted genes (e.g., *ETS2*) contributes to the advanced cancer phenotype awaits further investigation.

### *Distinct roles of ETV1 and ERG in prostate tumorigenesis have implications for prostate cancer therapy*

Our analysis of gene expression and patient outcome data sets underscores the relevance of distinct features of ETV1-regulated pathways to invasive adenocarcinoma progression. ETV1-defined, but not ERG-defined, gene sets are associated with high Gleason score and metastasis (Fig. 7E; Supplemental Fig. S18C). Our observation that *ERG* expression does not correlate with the worse outcome is consistent with a recent meta-analysis describing no association of ERG with Gleason score, clinical outcome, or recurrence of the disease including 62 cohorts (Pettersson et al. 2012). Of note, ERG mRNA and protein level analysis (Markert et al. 2011; Pettersson et al. 2012) showed that *TMPRSS2-ERG* fusion status does not always correlate with the *TMPRSS2-ERG* transcriptional signature or ERG protein level in prostate cancer patients. Accordingly, most recent clinical studies have supported high ERG expression levels as a favorable prognosis biomarker (Bismar et al. 2012; Kimura et al. 2012; Suh et al. 2012). Consistent with our findings, however, *ETV1* expression at the transcript level has been associated with a greater Gleason score and recurrence of the disease (Attard et al. 2008b; Shin et al. 2009). Unfortunately, thus far, it has not been possible to study clinical relevance of ETV1 at the protein level due to the lack of satisfactory antibodies. Moreover, ETV1, rather than ERG, is among AR ChIP targets defined recently from primary CRPC patient samples (Sharma et al. 2013). Last, there is also a high overlap between ETV1-associated, castration-associated, and recurrent prostate tumor signatures (Supplemental Fig. S18E). Although *TMPRSS2-ETV1* fusions are only found in ~1%–2% of all prostate cancer cases, prostate tumors with elevated *ETV1* expression (5%–10% of all cases) are enriched in advanced disease (Fig. 7A,B), suggesting that the ETV1-driven oncogenic program may be selected for during prostate cancer progression.

In summary, our data suggest that ETV1 is a novel marker of aggressive prostate cancer, and the oncogenic program it drives may be an important therapeutic target for treating advanced prostate cancer. Metabolic enzymes (such as HSD17B7) that are regulated by ETV1 may be explored as therapeutic targets. Moreover, ETS factors have been described to modulate the Ras/MAPK pathway (Hollenhorst et al. 2011). Interestingly, ETV1 overexpression, but not that of ERG, is associated with Ras/MAPK activity in a range of tumors, including ETV1-dependent melanoma and gastrointestinal stromal tumor, where ETV1 is a master regulator of lineage (Chi et al. 2010; Jane-Valbuena et al. 2010). These observations raise the possibility that MAPK inhibitors may be explored to target ETV1-overexpressing tumors. In conclusion, our study suggests that tumors characterized by an ETV1 expression signature through either translocation or other mechanisms represent a distinct biological entity associated with aggressive prostate cancer. Future research should focus on exploring novel therapeutic approaches for this entity.

## Materials and methods

### *Mouse lines*

*Tmprss2-ETS* conditional knock-in mice were generated by standard gene targeting. *Pb-Cre* (*Pb-Cre4*) transgenic mice were acquired from the Mouse Models of Human Cancers Consortium (MMHCC) repository. *Pten* conditional knockout mice (*Pten<sup>L</sup>*) and *Pb-AR* [FVB-Tg(Pbsn-Ar\*E231G)] transgenic mice were acquired from JAX. *Pten<sup>+/-</sup>* mice were generated by crossing *Pten<sup>L/+</sup>* mice to *Gata1-Cre* mice. All studies were approved by the Institutional Animal Care and Use Committee (IACUC).

### *Pathology, immunostaining, and flow cytometry*

Standard protocols were followed.

### *Cell lines*

Cell lines were obtained from American Type Culture Collection and cultured accordingly. ERG or ETV1 overexpression and silencing experiments were performed by standard protocols.

### *RT-PCR*

Real-time RT-PCR was performed according to standards protocols. All primer sequences are listed in Supplemental Table S2.

### *Gene expression microarray analysis*

RWPE-1 stable cell clones (R.ERG, R.ETV1, and R.CTL) were grown under normal conditions. VCaP and LNCaP cells, 24 h after ERG or ETV1 RNAi, respectively, were grown in hormone-depleted conditions for 2 d, and then in the presence or absence of 10 nM DHT for 16 h. Mouse primary prostate cells were FACS-sorted and processed according to standard procedures. Affymetrix HG133 plus 2.0 or Mouse Genome 430 2.0 expression arrays were used for human or mouse samples, respectively. Gene Pattern software (Reich et al. 2006) was used for data normalization, extraction of expression values, and generation of GTC files for GSEA (Subramanian et al. 2005). A bidimensional comparison plot was used to compare differentially expressed genes ( $P < 0.05$  by two-tailed *t*-test) in RWPE-1 cells upon overexpression of either ETV1 or ERG.

### *ChIP and ChIP-on-chip*

BioChIP-chip for ETV1 was performed as described (Kim et al. 2008), and conventional ChIP-chip reaction for ERG was as described (Kim et al. 2004). Affymetrix Human Promoter 1.0R array hybridization was performed at the Dana-Farber Cancer Institute Microarray Core Facility. Peak identification was calculated by MAT score (Johnson et al. 2006). For ChIP-PCR experiments, conventional ChIP reactions were performed. Antibodies used were as follows: anti-AR (N20X), anti-ERG (C17X) and anti-rabbit IgG from Santa Cruz Biotechnology, and anti-ETV1 kindly provided by Dr. Litovchick. The online DAVID functional annotation tool (Huang et al. 2009) and the IPA tool (Ingenuity Systems, Inc.) were used to determine the enrichment for all "FAT" GO terms and canonical pathways/networks in each gene set.

### *Patient tumor data analysis*

Gene sets associated with indolent and aggressive prostate cancer were extracted from the Swedish, MSKCC, Sharma, and



Glinsky cohorts (Glinsky 2004; Setlur et al. 2008; Taylor et al. 2010; Sharma et al. 2013) and analyzed for their mutual overlap between tumor cohort-derived signatures (differentially expressed genes: fold change, >1.5; false discovery rate [FDR], <0.05) and ERG- and ETV1-associated gene sets obtained in our studies. The overlap between gene sets was represented by a connectivity network, where the width of the connector edge was  $-\log_{10}(P\text{-value})$ . The  $P$ -value was derived from a hypergeometric distribution by using Fisher exact test to analyze the significance of the mutual overlap. Cytoscape software version 2.8 (Cline et al. 2007) was used for the visualization of gene sets overlapping the network.

### Statistics

All statistics were based on a Student's  $t$ -test, unless otherwise indicated. Dot plots and histograms show data means, and error bars are standard error of the mean (SEM). All statistics were performed using the data analysis package within Microsoft Excel or the analysis tool within GraphPad Prism 5.0. Kaplan-Meier survival analysis was performed using GraphPad Prism 5.0.

### Steroid metabolism measurement

Steroids from RWPE-1 and androgen-deprived LNCaP cells were extracted following Lemmen et al. (2002) and quantified by LC/MS at the Harvard FAS Center for Systems Biology.

### Testosterone measurement

The intraprostatic testosterone levels were measured by a mouse testosterone ELISA kit (Calbiotech) based on the manufacturer's instructions. Briefly, mouse prostates were microdissected in cold PBS and lysed in RIPA buffer. Testosterone levels were calculated as the total amount per gram of total protein.

### Accession number

The Gene Expression Omnibus accession number is GSE39388.

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